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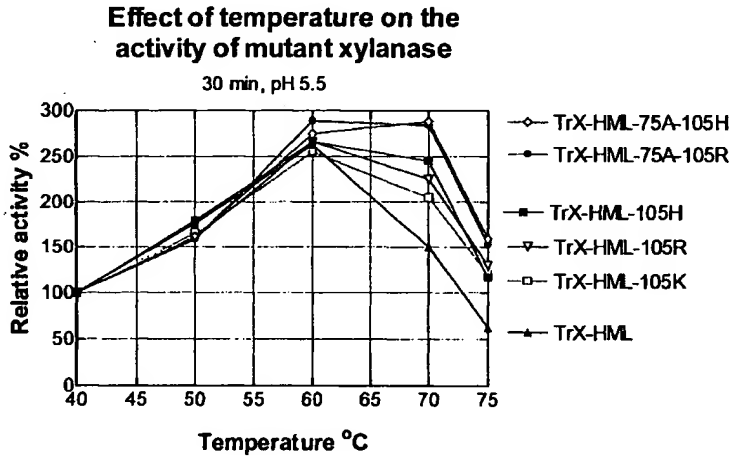
(54) Title: MODIFIED XYLANASES EXHIBITING INCREASED THERMOPHILICITY AND ALKALOPHILICITY



(57) Abstract: The present invention pertains to modified xylanase enzymes that exhibit increased thermostability and alkalophilicity, when compared with their native counterparts. Several modified xylanases exhibiting these properties are disclosed including xylanases with at least one modification at amino acid position (10, 27, 29, 75, 104, 105, 125, 129, 132, 135, 144, 157, 161, 162, 165) or a combination thereof. Also included within the present invention is a modified xylanase that comprise at least one substituted amino acid residue and that may be characterized as having a maximum effective temperature (MET) between about 69 °C to about 78 °C, wherein the modified xylanase is a Family 11 xylanase obtained from a *Trichoderma* sp.. The present invention also includes a modified Family 11 xylanase obtained from a *Trichoderma* sp. characterized as having a maximum effective pH (MEP) between about 5.8 to about 7.6. Modified xylanases characterized as having a MET between about 69 °C to about 78 °C and a MEP between about 5.8 to about 7.6 are also disclosed.

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MODIFIED XYLANASES EXHIBITING INCREASED THERMOPHILICITY AND ALKALOPHILICITY

The present invention relates to modified xylanases. More specifically, the invention relates to modified xylanases with improved performance at conditions of high temperature and pH.

BACKGROUND OF THE INVENTION

Xylanases are a group of enzymes with wide commercial utility. A major application of xylanases is for pulp biobleaching in the production of paper. In addition, xylanases have been used as clarifying agents in juices and wines, as enzymatic agents in the washing of precision devices and semiconductors (e.g. U.S. Pat. No. 5,078,802), and they are also used for improving digestibility of poultry and swine feed.

In the manufacturing of pulp for the production of paper, fibrous material is subjected to high temperatures and pressures in the presence of chemicals. This treatment converts the fibers to pulp and is known as pulping. Following pulping, the pulp is bleached. Xylanase enzymes are used to enhance the bleaching of the pulp. The xylanase treatment allows subsequent bleaching chemicals such as chlorine, chlorine dioxide, hydrogen peroxide, or combinations of these chemicals to bleach pulp more efficiently. Pretreatment of pulp with xylanase increases the whiteness and quality of the final paper product and reduces the amount of chlorine-based chemicals which must be used to bleach the pulp. This in turn decreases the chlorinated effluent produced by such processes.

The most important chemical pulping process is kraft pulp. For kraft pulp, following pulping, and prior to the treatment of pulp with xylanase, the pulp is at about a temperature of 55-70°C and at a highly alkaline pH (e.g. Nissen et al., 1992). A drawback of many commercially available wild-type xylanases, is that these enzymes

exhibit an acidic pH optimum and a temperature optimum of about 55°C. Therefore, in order to effectively utilize xylanases for bleaching applications, the pulp must be acidified to a pH approximating the optimal pH for the specific xylanase used. In addition, the hot pulp must be cooled to a temperature close to the optimal temperature for enzymatic activity of the selected xylanase. Decreasing pulp temperatures for xylanase treatment decreases the efficiency of the subsequent chemical bleaching. Acidification of pulp requires the use of large quantities of acids. Further, the addition of acids leads to corrosion, which lessens the lifetime of process equipment. Thus, xylanases optimally active at temperatures and pH conditions approximating the conditions of the pulp would be useful and beneficial in pulp manufacturing.

Xylanases which exhibit greater activity at higher temperatures could be used to treat pulp immediately following the pulping process, without the need to cool the pulp. Similarly, xylanases which exhibit greater activity at higher pH conditions would require less or no acid to neutralize the pulp. The isolation of, or the genetic manipulation of, xylanases with such properties would provide several advantages and substantial economic benefits within a variety of industrial processes.

Several approaches directed towards improving xylanase for use in pulp-bleaching within the prior art include the isolation of thermostable xylanases from extreme thermophiles that grow at 80-100°C, such as *Caldocellum saccharolyticum*, *Thermatoga maritima* and *Thermatoga sp.* Strain FJSS-B.1 (Lüthi et al. 1990; Winterhalter et al. 1995; Simpson et al. 1991). However, these thermostable xylanase enzymes are large, with molecular masses ranging from 35-120 kDa (320-1100 residues), and exhibit a reduced ability to penetrate the pulp mass compared with other smaller xylanases which exhibit better accessibility to pulp fibers. In addition, some of the extremely thermophilic xylanases, such as *Caldocellum saccharolyticum* xylanase A, exhibit both xylanase and cellulase activities (Lüthi et al. 1990). This additional cellulolytic activity is undesirable for pulp bleaching, due to its detrimental effect on cellulose, the bulk material in paper. Furthermore, hyper-thermostable xylanase enzymes

which function normally at extremely high temperatures have low specific activities at temperatures in the range for optimal pulp bleaching (Simpson et al. 1991).

A number of xylanases have been modified by protein engineering to improve their properties for industrial applications. For instance, U.S. 5,759,840 (Sung et al.), and U.S. 5,866,408 (Sung et al.) disclose mutations in the N-terminal region (residues 1-29) of *Trichoderma reesei* xylanase II (TrX). Three mutations, at residues 10, 27 and 29 of TrX, were found to increase the enzymatic activity of the xylanase enzyme at elevated temperatures and alkaline pH conditions.

U.S. 5,405,769 (Campbell et al.), discloses modification of *Bacillus circulans* xylanase (BcX) using site-directed mutagenesis to improve the thermostability of the enzyme. The site specific mutations include replacing two amino acids with Cys residues to create intramolecular disulfide bonds. In addition, specific residues in the N-terminus of the enzyme were mutated which were also found to further improve the thermostability of the enzyme. In *in vitro* assays, the disulfide mutants showed thermostability at 62° C, an improvement of 7° C over the native BcX xylanase enzyme. However, these thermostable disulfide mutants showed no gain in thermophilicity in laboratory assays in subsequent studies (Wakarchuck et al., 1994). Mutations T3G (i.e. threonine at position 3 replaced with Gly; BcX xylanase amino acid numbering), D4Y(F) and N8Y(F) near the N-terminus of the BcX xylanase enzyme provided thermostability to 57° C, an increase of 2° C over the native BcX (U.S. 5,405,769). However, the use of these enzymes within industrial applications still requires cooling and acidification of pulp following pretreatment, prior to enzyme addition. Therefore, further increases in thermostability, thermophilicity and pH optima are still required.

There is a need in the prior art to obtain novel xylanases which exhibit increased enzymatic activity at elevated temperatures and pH conditions, suitable for industrial use. It is an object of the invention to overcome drawbacks in the prior art.

The above object is met by the combination of features of the main claim, the sub-claims disclose further advantageous embodiments of the invention.

SUMMARY OF THE INVENTION

The present invention relates to modified xylanases. More specifically, the invention relates to modified xylanases with improved performance at conditions of high temperature and pH.

This invention relates to a modified xylanase comprising at least one substituted amino acid residue at a position selected from the group consisting of amino acid 75, 104, 105, 125, 129, 132, 135, 144, 157, 161, 162, and 165 the position determined from sequence alignment of the modified xylanase with *Trichoderma reesei* xylanase II amino acid sequence defined in SEQ ID NO:16. Preferably, the modified xylanase exhibits improved thermophilicity, alkalophilicity, or a combination thereof, in comparison to a corresponding native xylanase.

The present invention also provides for the modified xylanase as defined above wherein the at least one substituted amino acid residue is at position 75. Preferably the substituted amino acid is selected from the group consisting of Ala, Cys, Gly, and Thr.

The present invention also embraces modified the modified xylanase as defined above and further comprising a His at position 10, Met at position 27 and Leu at position 29.

According to the present invention there is also provided a modified xylanase comprising a substituted amino acid residue at position 105, the position determined from sequence alignment with *Trichoderma reesei* xylanase II amino acid sequence defined in SEQ ID NO:16. Preferably, the substituted amino acid is selected from the group consisting of His, Lys, and Arg. The present invention also pertains to the modified xylanase just defined further comprising a His at position 10, Met at position 27 and Leu at position 29. The invention also includes the modified xylanase just defined further comprising a substituted amino acid residue at position 75.

This invention also includes a modified xylanase comprising a His at position 10, a Met at position 27, a Leu at position 29, a non-polar amino acid at positions 75 and 125, a non-polar amino acid at positon 104, a polar amino acid at position 105, and an acidic amino acid at position 129. Preferably, the amino acid at postion 75 is Ala, the amino acid at position 125 is selected from the group consisting of Ala, Cys, Gly, and Thr, the amino acid at position125 is Glu. The amino acid at position 105 is selected from the group consisting of His, Lys, and Arg, and the amino acid residue at position 104 is Pro.

This invention further relates to a modified xylanase comprising a His at position 10, a Met at position 27, a Leu at position 29, a non-polar amino acid at positions 75 and 125, a polar amino acid at positions 105, 132 and 135, and an acidic amino acid at position 129. Furthermore, the modified xylanase as just defined may include a polar amino acid at position144.

This invention includes a modified xylanase comprising a His at position 10, a Met at position 27, a Leu at position 29, a non-polar amino acid at positions 75 and 125, a polar amino acid at positions 105, 132, 135, 144, 157, 161, 162 and 165, and an acidic amino acid at position 129.

This invention embraces a modified xylanase comprising a His at position 10, a Met at position 27, a Leu at position 29, a non-polar amino acid at positions 75 and 125, a polar amino acid at positions 105, 132, 135, 157, 161, 162 and 165, and an acidic amino acid at position 129.

This invention also pertains to a modified xylanase comprising a His at position 10, a Met at position 27, a Leu at position 29, a non-polar amino acid at positions 75 and 125, and a polar amino acid at positions 105,135, 144, 157, 161, 162 and 165.

The present invention is also directed to the modified xylanases, as defined above, wherein the modified xylanases are derived from a Family 11 xylanase, preferably a *Trichoderma reesei* xylanase.

The present invention pertains to a modified xylanase comprising at least one substituted amino acid residue, wherein the modified xylanase is characterized as having a maximum effective temperature (MET) between about 69°C to about 78°C, and wherein the modified xylanase is a Family 11 xylanase obtained from a *Trichoderma* sp.. Preferably, the MET is between about 70° to about 75°C

This invention also includes a modified xylanase comprising at least one substituted amino acid residue, wherein the modified xylanase is characterized as having a maximum effective pH (MEP) between about pH 5.8 to about pH 7.6, and wherein the modified xylanase is a Family 11 xylanase obtained from a *Trichoderma* sp.. Preferably, the MEP is between about pH 6.5 to about pH 7.4.

The present invention is directed to a modified xylanase comprising at least one substituted amino acid residue, wherein the modified xylanase is characterized as having a maximum effective temperature (MET) between about 69°C to about 78°C, and a maximum effective pH (MEP) between about pH 5.8 to about pH 7.6. Prefereably, the MET is between about 70° to about 75°C, and the MEP is between about pH 6.5 to about pH 7.4.

The present invention also relates to a modified xylanase selected from the group consisting of:

TrX-75A
TrX-157D-161R-162H-165H;
TrX-HML-75A;
TrX-HML-105H;
TrX-HML-105R;
TrX-HML-105K;
TrX-HML-75A-105H;
TrX-HML-75A-105R;
TrX-HML-75C-105R;

TrX-HML-75G-105R;
TrX-HML-75T-105R
TrX-HML-125A;
TrX-HML-125A-129E;
TrX-HML-75G-105R-125A-129E (TrX-HML-GRAE);
TrX-HML-75A-105H-125A-129E (TrX-HML-AHAE);
TrX-HML-75G-105H-125A-129E (TrX-HML-GHAE);
TrX-HML-75A-105R-125A-129E (TrX-HML-ARAE);
TrX-HML-75G-104P-105R-125A-129E (TrX-HML-GPRAE);
TrX-HML-75G-104P-105H-125A-129E (TrX-HML-GPHAE);
TrX-HML-AHAE-RR;
TrX-HML-AHAE-RRR;
TrX-HML-AHAE-RRR-DRHH;
TrX-HML-AHA-RR-DRHH; and
TrX-HML-AHAE-RR-DRHH.

According to the present invention, there is provided a modified xylanase comprising at least one substituted amino acid residue, and characterized as having a maximum effective temperature (MET) between about 69°C to about 78°C, wherein the modified xylanase is a Family 11 xylanase obtained from a *Trichoderma* sp.. Furthermore the present invention relates to a modified Family 11 xylanase obtained from a *Trichoderma* sp. characterized as having a MET between about 70° to about 75°C. The present invention also includes the modified Family 11 xylanase obtained from a *Trichoderma* sp. characterized as having a MET between about 69°C to about 78°C and a maximum effective pH (MEP) between about 5.8 to about 7.6. This invention also pertains to the modified xylanase as just defined, wherein the MEP is between about 6.5 to about 7.4.

The present invention is directed to the use of the modified xylanase as defined above in an industrial process. Also included is an industrial process, wherein the

industrial process comprises bleaching of pulp, processing of precision devices, or improving digestibility of poultry and swine feed.

This summary of the invention does not necessarily describe all necessary features of the invention but that the invention may also reside in a subcombination of the described features.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other features of the invention will become more apparent from the following description in which reference is made to the appended drawings wherein:

FIGURE 1 shows an amino acid sequence alignment among Family 11 xylanases. The amino acid numbering is compared with *Trichoderma reesei* xylanase II (Tr2) as indicated at the top of the sequences. The residues at position 75 and 105 (relative to Tr2) are in italic and indicated with an asterisk. The amino acids common to at least 75% of the listed Family 11 xylanases are indicated in bold. The residues common to all Family 11 xylanases are underlined. For xylanases with a cellulose-binding domain, only the catalytic core sequences are presented.

FIGURE 2 shows the nucleotide sequence of TrX xylanase (SEQ ID NO:39), and the synthetic oligonucleotides used to construct the sequence encoding the *Trichoderma reesei* xylanase II enzyme (TrX) in the plasmid pTrX.

FIGURE 3 shows the effect of temperature on the enzymatic activity of modified xylanase TrX-75A, compared with TrX, at pH 5.5 during 30 min incubations. The data are normalized to the activity observed at 40°C.

FIGURE 4 shows the effect of temperature on the enzymatic activity of modified xylanases TrX-HML, TrX-HML-75A, TrX-HML-105H and TrX-HML-75A-105H, at pH 5.5 during 30 min incubations. The data are normalized to the activity observed at 40° C.

FIGURE 5 shows the effect of temperature on the enzymatic activity of modified xylanases TrX-HML, TrX-HML-105K, TrX-HML-105R, TrX-HML-105H, TrX-HML-75A-105R and TrX-HML-75A-105H at pH 5.5 during 30 min incubations. The data are normalized to the activity observed at 40° C.

FIGURE 6 shows the effect of temperature on the enzymatic activity of modified xylanases TrX-HML, TrX-HML-105R, TrX-HML-75T-105R, TrX-HML-75G-105R, TrX-HML-75A-105R and TrX-HML-75C-105R at pH 5.5 during 30 min incubations. The data are normalized to the activity observed at 40° C.

FIGURE 7 shows the effect of temperature on the enzymatic activity of modified xylanase enzyme TrX-HML, TrX-HML-125A, TrX-HML-125A129E and TrX-HML-75G-105R-125A129E (TrX-HML-GRAE) at pH 5.5 during 30 min incubations. The data are normalized to the activity observed at 40°C.

FIGURE 8 shows the effect of temperature on the enzymatic activity of modified xylanase enzymes:

TrX-HML;

TrX-HML-105H;

TrX-HML-75A-105H-125A129E (TrX-HML-AHAE);

TrX-HML-75G-105H-125A129E (TrX-HML-GHAE); and

TrX-HML-75A-105R-125A129E (TrX-HML-ARAE)

at pH 5.5 during 30 min incubations. The data are normalized to the activity observed at 40°C.

FIGURE 9 shows the effect of temperature on the enzymatic activity of modified xylanase enzymes:

TrX-HML-75G-104P-105R-125A129E (TrX-HML-GPRAE);

TrX-HML-75G-104P-105H-125A129E (TrX-HML-GPHARE); and

TrX-HML-75G-105R-125A129E (TrX-HML-GRAE)

at pH 5.5 during 30 min incubations. The data are normalized to the activity observed at 40°C.

FIGURE 10 shows the pH profile of modified xylanase enzyme TrX-75A compared with native TrX, over pH 4.0 - 6.5, at 55° C during 30 min incubation. The data are normalized to the pH exhibiting optimal activity for each enzyme.

FIGURE 11 shows the pH profiles of modified xylanases TrX-HML, TrX-HML-75A, TrX-HML-105H and TrX-HML-75A-105H over pH 4-7, at 65°C during 30 min incubation. The data are normalized to the pH exhibiting optimal activity for each enzyme.

FIGURE 12 shows the pH profile of modified xylanases TrX-HML, TrX-HML-105K, TrX-HML-105R, TrX-HML-105H and TrX-HML-75A-105H over pH 4-7, at 65° C during 30 min incubation. The data are normalized to the pH exhibiting optimal activity for each enzyme.

FIGURE 13 shows the pH profile of modified xylanases:

TrX-HML;
TrX-HML-105R;
TrX-HML-75T-105R, TrX-HML-75G-105R;
TrX-HML-75A-105R; and
TrX-HML-75C-105R

over pH 4-7, at 65° C during 30 min incubations. The data are normalized to that observed at the pH for optimal activity of the enzyme.

FIGURE 14 shows the pH profile of modified xylanases:

TrX-HML;
TrX-HML-105H;
TrX-HML-75A-105H;
TrX-HML-75A-105H-125A129E (TrX-HML-AHAE); and
TrX-HML-75G-105H-125A129E (TrX-HML-GHAE)

over pH 4-7, at 65°C during 30 min incubations. The data are normalized to the pH exhibiting optimal activity for each enzyme.

FIGURE 15 shows the effect of temperature on the enzymatic activity of modified xylanase TrX-157D-161R-162H-165H, compared with TrX, at pH 5.5 during 30 min incubations. The data are normalized to the activity observed at 40°C.

FIGURE 16 shows the pH profile of modified xylanase enzyme TrX-157D-161R-162H-165H compared with native TrX, over pH 4.0 - 6.5, at 55° C during 30 min incubation. The data are normalized to the pH exhibiting optimal activity for each enzyme.

FIGURE 17 shows the effect of temperature on the enzymatic activity of modified xylanases:

TrX-HML-AHAE-RR; and

TrX-HML-AHAE-RRR

at pH 5.5 during 30 min incubations. The data are normalized to the activity observed at 40° C.

FIGURE 18 shows the effect of temperature on the enzymatic activity of modified xylanases:

TrX-HML-AHA-RR-DRHH;

TrX-HML-AHAE-RR-DRHH; and

TrX-HML-AHAE-RRR-DRHH

at pH 5.5 during 30 min incubations. The data are normalized to the activity observed at 40° C.

FIGURE 19 shows the effect of temperature on the enzymatic activity of modified xylanases:

TrX;

TrX-HML

TrX-HML-AHAE

TrX-HML-AHAE-RRR-DRHH;

TrX-HML-AHA-RR-DRHH; and

TrX-HML-AHAE-RR-DRHH

at pH 5.5 during 30 min incubations. The data are normalized to the activity observed at 40° C.

FIGURE 20 shows the maximum effective temperature (MET) and maximum effective pH (MEP) values of several of the modified enzymes of the present invention. The MET and MEP are the highest temperature and pH, respectively, at which a xylanase exhibits at least 80% of its optimal activity (using xylan as a substrate; see method for complete details of assays). These data points were obtained from the data presented in Figures 5 to 14.

DESCRIPTION OF PREFERRED EMBODIMENT

The present invention relates to modified xylanases. More specifically, the invention relates to modified xylanases with improved performance at conditions of high temperature and pH.

The following description is of a preferred embodiment by way of example only and without limitation to the combination of features necessary for carrying the invention into effect.

The mechanism by which xylanases facilitate bleaching of pulp is not fully understood. It has been postulated that the coloured lignin is connected to crystalline cellulose through xylan and xylanase enzymes facilitate bleaching of pulp by hydrolysing xylan, releasing coloured lignin in the pulp. Modified xylanases, as outlined herein, may be used for the purposes of bleaching pulp or other applications requiring activities at temperatures and pH above that of the wild-type enzyme. For the biobleaching of pulp, the preferred xylanase is derived from a xylanase classified in Family 11 (see Table 1), however, the modifications disclosed herein need not be limited to only Family 11 xylanases and may include other xylanase enzymes.

Family 11 xylanase enzymes are a group of small enzymes of relatively low molecular mass (approximately 20 kDa, and about 200 amino acid residues. The small size associated with Family 11 xylanases permits ready penetration of the pulp mass. Furthermore, Family 11 xylanases are free of cellulase activity.

One aspect of the present invention is directed to a modified Family 11 xylanase obtained from a *Trichoderma* sp. comprising at least one substituted amino acid residue, and characterized as having a maximum effective temperature (MET) between about 69°C to about 78°C. Preferably, the modified xylanase is characterized as having a MET between about 70° to about 75°C. This invention also includes a modified xylanase comprising at least one substituted amino acid residue, and is characterized as having a

maximum effective pH (MEP) between about 5.8 to about 7.6. Preferably, the MEP is between about 6.5 to about 7.4.

This invention also pertains to a modified xylanase obtained from *Trichoderma*, comprising at least one substituted amino acid, and characterized as having a maximum effective temperature (MET) between about 69°C to about 78°C, and a maximum effective pH (MEP) is between about 5.8 to about 7.6. Preferably the MET is between about 70°to about 75°C, and the MEP is between about 6.5 to about 7.4.

By "maximum effective temperature" or "MET" it is meant the highest temperature at which a xylanase exhibits at least 80% of its optimal activity. This test is typically carried out using xylan as a substrate at pH 5.5, and for a 30 min period. Results from assays used to characterize modified xylanases are presented in Figures 3 to 9 and involved a 30 min incubation at pH 5.5. A summary of the MET of several enzymes of the present invention, determined from Figures 3 to 9 is presented in Figure 20. Experiments demonstrate that the MET of a xylanase differs on different substrates. Therefore, it is to be understood that with different substrates, different MET values will be obtained (data not presented). For the purposes of evaluating xylanases of the present invention, the xylan substrate is used (see examples 3 and 4).

By "maximum effective pH" or "MEP" it is meant the highest pH at which a xylanase exhibits at least 80% of its optimal activity. This test is carried out using xylan as a substrate, at 65°C, and for a 30 min period. Results from assays used to characterize modified xylanases are presented in Figures 10 to 14 and 16 to 19 and involved a 30 min incubation at 65°C. A summary of the MEP of several enzymes of the present invention is presented in Figure 20. Experiments demonstrate that the MEP of a xylanase differs on different substrates. For example, on kraft pulp prepared from soft wood or hardwood, a MEP of 8.5 has been observed (data not presented). Therefore, it is to be understood that with different substrates, different MEP values will be obtained. For the purposes of evaluating xylanases of the present invention, the xylan substrate is used (see examples 4 and 5).

TABLE 1. Family 11 xylanase enzymes

Microbe	Xylanase	SEQ ID NO
Aspergillus niger	Xyn A	SEQ ID NO: 1
Aspargillus awamori var.kawachi	Xyn B	SEQ ID NO: 19
Aspergillus kawachii	Xyn C	–
Aspergillus tubigensis	Xyn A	SEQ ID NO: 2
Bacillus circulans	Xyn A	SEQ ID NO: 3
Bacillus pumilus	Xyn A	SEQ ID NO: 4
Bacillus subtilis	Xyn A	SEQ ID NO: 5
Cellulomonas fimi	Xyn D	–
Chainia spp.	Xyn	–
Clostridium acetobutylicum	Xyn B	SEQ ID NO: 6
Clostridium stercorarium	Xyn A	SEQ ID NO: 7
Fibrobacter succinognees	Xyn II	SEQ ID NO: 18
Neocallimasterix patriciarum	Xyn A	–
Nocardiopsis dassonvillei	Xyn II	–
Ruminococcus flavefaciens	Xyn A	SEQ ID NO: 8
Schizophyllum cimmune	Xyn	SEQ ID NO: 9
Streptomyces lividans	Xyn B	SEQ ID NO: 10
Streptomyces lividans	Xyn C	SEQ ID NO: 11
Streptomyces sp. No. 36a	Xyn	SEQ ID NO: 12
Streptomyces thermoviolaceus	Xyn II	–
Thermomonospora fusca	Xyn A	SEQ ID NO: 13
Thermomyces lanuginosus	Xyn	SEQ ID NO: 20
Trichoderma harzianum	Xyn	SEQ ID NO: 14
Trichoderma reesei	Xyn I	SEQ ID NO: 15
Trichoderma reesei	Xyn II	SEQ ID NO: 16
Trichoderma viride	Xyn	SEQ ID NO: 17

Family 11 xylanases share extensive amino acid sequence similarity (Figure 1). Structural studies of several Family 11 xylanases indicate that Family 11 xylanases from bacterial and fungal origins share the same general molecular structure (U.S. 5,405,769; Arase et al 1993). In addition, most Family 11 xylanases identified so far exhibit three types of secondary structure, including beta-sheets, turns and a single alpha helix. The helix of *Trichoderma reesei* xylanase II enzyme encompasses the region from amino acid 151 to amino acid 162 (Torronen et. al. 1995).

A xylanase is classified as a Family 11 xylanase if it comprises amino acids common to other Family 11 xylanases, including two glutamic acid (E) residues which may serve as catalytic residues. The glutamic acid residues are found at positions 86 and 177 (see Figure 1; based on Tr2 (*Trichoderma reesei* xylanase II enzyme) amino acid numbering).

Most of the Family 11 xylanases identified thus far are mesophilic and have low-molecular masses (20 kDa). However, this family also includes at least two thermostable xylanases of higher molecular mass, *Thermomonospora fusca* xylanase A (TfX-A) of 296 amino acids and a molecular mass of approximately 32 kDa (Irwin et. al., 1994); Wilson et al. 1994, WO 95/12668) and *Clostridium stercorarium* xylanase A of 511 amino acids and a molecular mass of approximately 56 Kda. The *Clostridium stercorarium* xylanase A enzyme exhibits maximum activity at a temperature of 70° C (Sakka et al.,1993).

The large thermostable Family 11 xylanases differ from the small mesophilic enzymes by the possession of a hydrophobic cellulose-binding domain (CBD) in the extended C-terminus of the enzyme. The TfX-A enzyme is composed of a catalytic core sequence of 189 residues common to all Family 11 xylanases, and a cellulose binding domain of 107 residues. The larger *C. stercorarium* xylanase A has 2 copies of the cellulose binding domain.

Site-directed mutagenesis has been used in the present invention to produce mutations in xylanases which render the enzyme more thermophilic and alkalophilic

compared to the native enzyme. Preferably, the mutant xylanase is one derived from a Family 11 xylanase. More preferably, the mutant xylanase of the present invention comprises a mutant *Trichoderma reesei* xylanase II enzyme.

Therefore, it is considered within the scope of the present invention that xylanases, including Family 11 xylanases for example but not limited to *Trichoderma reesei* xylanase II, *Trichoderma reesei* xylanase I, *Trichoderma viride* xylanase, *Streptomyces lividans* xylanase B and *Streptomyces lividans* xylanase C, may be modified following the general approach and methodology as outlined herein. It is also considered within the scope of the present invention that non-Family 11 xylanases may also be modified following the general principles as described herein in order to obtain a xylanase enzyme that exhibits thermophilicity and alkalophilicity.

By the term "thermophilicity" it is meant that an enzyme is active, or more active, at a higher temperature when compared with the activity of another enzyme when all other conditions remain constant. For example, xylanase 1 exhibits increased thermophilicity compared to xylanase 2 if xylanase 1 is capable of, or is more active in, hydrolysing xylan at a higher temperature than xylanase 2, under identical conditions using the same substrate. As most xylanases are effective at a higher temperature when hydrolysing pure xylan rather than pulp, comparative analysis should be made using the same substrate. Quantitative measures of thermophilicity referred to herein use pure xylan substrates unless otherwise indicated.

By "thermostability" it is meant the ability of an enzyme to be stored or incubated at high temperature conditions, typically in the absence of substrate, and then exhibit activity when returned to standard assay conditions. For example, xylanase 1 is said to display increased thermostability compared to xylanase 2 if xylanase 1 retains a greater amount of activity than xylanase 2 after being maintained at a certain temperature (typically a higher temperature), for example but not limited to, 70° C for 24 hours, followed by assay at a lower temperature. In contrast to thermophilicity, thermostability

relates to the remaining enzyme activity following an incubation in the absence of substrate.

These use of these two terms (thermophilicity and thermostability) has been confused within the prior art as they have been used interchangeably. However, the use of the terms as defined herein is consistent with the usage of the terms in the art (Mathrani and Ahring, 1992).

By "alkalophilicity" it is meant that an enzyme is active, or more active, at a higher pH when compared with the activity of another enzyme when all other conditions remain constant. For example, xylanase 1 exhibits increased alkalophilicity compared to xylanase 2 if xylanase 1 is capable of hydrolysing xylan at a higher pH than xylanase 2. Typically alkalophilicity relates to enzyme activity in the presence of xylan substrate.

By "TrX numbering" it is meant the numbering associated with the position of amino acids based on the amino acid sequence of TrX (Xyn II - Table 1; Tr2 - Figure 1; SEQ ID NO:16). As disclosed below and as is evident upon review of Figure 1, Family 11 xylanases exhibit a substantial degree of sequence similarity. Therefore, by aligning the amino acids to optimize the sequence similarity between xylanase enzymes and by using the amino acid numbering of TrX as the basis for numbering, the positions of amino acids within other xylanase enzymes can be determined relative to TrX.

By modified xylanase, it is meant the alteration of a xylanase molecule using techniques that are known to one of skill in the art. These techniques include, but are not limited to, site directed mutagenesis, cassette mutagenesis, random mutagenesis, synthetic oligonucleotide construction, cloning and other genetic engineering techniques.

As described in more detail herein, several mutant xylanases have been prepared that exhibit increased thermophilicity, alkalophilicity and thermostability when compared

to native xylanase. A list of several of mutants, which is not to be considered limiting in any manner, is presented in Table 2.

Furthermore, the present is directed to a modified Family 11 xylanase obtained from a *Trichoderma* sp. that comprises at least one substituted amino acid residue, and characterized as having a maximum effective temperature (MET) between about 69°C to about 78°C. Preferably, the modified xylanase is characterized as having a MET between about 70° to about 75°C. This invention also pertains to a modified xylanase obtained from *Trichoderma*, comprising at least one substituted amino acid, and characterized as having a maximum effective pH (MEP) between about 5.8 to about 7.6. Preferably the MEP is between about 6.5 to about 7.4. This invention also pertains to a modified xylanase obtained from *Trichoderma*, comprising at least one substituted amino acid, and characterized as having a maximum effective temperature (MET) between about 69°C to about 78°C, and a maximum effective pH (MEP) is between about 5.8 to about 7.6. Preferably the MET is between about 70°to about 75°C, and the MEP is between about 6.5 to about 7.4.

Determination of the MET and MEP of a xylanase may be carried out as follows:

i) measure the temperature profile of a xylanase as outlined in Example 3. The temperatures for which at least 80% of the optimal (maximum) activity are determined, and the highest temperature is the MET;

ii) measure the pH profile of a xylanase as outlined in Example 4. The pH for which at least 80% of the optimal (maximum) activity is determined, and the highest pH is the MEP.

These values may then be plotted as shown in Figure 20.

Table 2: Modified xylanases

Xylanase	Description
TrX-75A	TrX with Ser at position 75 replaced with Ala (S75A)
TrX-105H	TrX with Leu at position 105 replaced with His (L105H)
TrX-HML	TrX with N10H, Y27M, and N29L (see U.S. 5,759,840)
TrX-HML-105H	TrX N10H, Y27M, N29L and L105H
TrX-HML-105K	TrX N10H, Y27M, N29L and L105K
TrX-HML-105R	TrX N10H, Y27M, N29L and L105R
TrX-HML-75A	TrX N10H, Y27M, N29L and S75A
TrX-HML-75A-105H	TrX N10H, Y27M, N29L, S75A, and L105H
TrX-HML-75A-105R	TrX N10H, Y27M, N29L, S75A and L105R
TrX-HML-75C-105R	TrX N10H, Y27M, N29L, S75C and L105R
TrX-HML-75G-105R	TrX N10H, Y27M, N29L, S75G and L105R
TrX-HML-75T-105R	TrX N10H, Y27M, N29L, S75T and L105R
TrX-HML-125A	TrX N10H, Y27M, N29L and Q125A
TrX-HML-125A129E	TrX N10H, Y27M, N29L, Q125A and I129E
TrX-HML-GRAE	TrX N10H, Y27M, N29L, S75G, L105R, Q125A and I129E
TrX-HML- AHAE	TrX N10H, Y27M, N29L, S75A, L105H, Q125A and I129E
TrX-HML-GHAE	TrX N10H, Y27M, N29L, S75G, L105H, Q125A and I129E
TrX-HML-ARAE	TrX N10H, Y27M, N29L, S75A, L105R, Q125A and I129E
TrX-HML-GPRAE	TrX N10H, Y27M, N29L, S75G, K104P, L105R, Q125A and I129E
TrX-HML-GPHAE	TrX N10H, Y27M, N29L, S75G, K104P, L105H, Q125A and I129E
TrX-HML-AHAE-RR	TrX N10H, Y27M, N29L, S75A, L105H, Q125A, I129E, A132R, and Y135R
TrX-HML-AHAE-RRR	TrX N10H, Y27M, N29L, S75A, L105H, Q125A, I129E, A132R, Y135R, and H144R
TrX-157D-161R-162H-165H	TrX N157D,Q161R, Q162H, and T165H
TrX-HML-AHAE-RRR-DRHH	TrX N10H, Y27M, N29L, S75A, L105H, Q125A, I129E, A132R, Y135R, H144R, N157D, Q161R, Q162H, and T165H

TrX-HML-AHA-RR-DRHH	TrX N10H, Y27M, N29L, S75A, L105H, Q125A, Y135R, H144R, N157D, Q161R, Q162H, and T165H
TrX-HML-AHAE-RR-DRHH	TrX N10H, Y27M, N29L, S75A, L105H, Q125A, I129E,Y135R, H144R, N157D, Q161R, Q162H, and T165H

Substitution at position 75 or 105 does not change the specific activity of the xylanase enzyme compared to that of native xylanase (see Table 4, Example 3). Similarly, mutations at position 157, 161, 162, and 165 do not change the specific activity on the modified xylanase.

Increasing the Thermophilicity of Xylanase

The mutant TrX-75A, bearing a single S75A mutation, showed greater enzymatic activity than the native TrX xylanase at 50, 55, 60 and 65°C (Figure 3). Further, the S75A mutation in the TrX-HML-75A mutant xylanase exhibited greater enzymatic activity than the TrX-HML parent xylanase at 70° C and 75° C (Figure 4). These results suggest that the S75A mutation improves the thermophilicity of TrX and TrX-HML xylanases.

The Ser to Ala mutation at position 75 (S75A) improves the thermophilicity for both TrX-75A and TrX-HML-75A xylanases in comparison to their native counterparts. The S75A mutation represents a change from a Ser amino acid bearing a side-chain which is relatively polar and hydrophilic to an Ala residue which bears a small and relatively nonpolar side-chain. Without wishing to be bound by theory, it is possible that replacing the polar serine amino acid with the smaller nonpolar Ala residue enhances intramolecular packing of the xylanase. The enhanced intramolecular packing of the tertiary structure of xylanase may in turn improve van der Waals interactions between closely positioned apolar substituents. The result of such improved intramolecular packing is an increase in the thermophilicity of the enzyme. In such cases, higher temperatures are required to denature and inactivate the mutant xylanase.

Substitution of position 157 with an acidic amino acid, and positions, 161, 162, and 165 with a basic amino acid, for example, but not limited to, replacing Asn at 157 with Asp (N157D), Ala at position 161 with Arg (A161R), Gln at position 162 with His (Q162H), and Thr at position165 with His (T165H) to produce TrX-157D-161R-162H-165H may result in a slight increase in the thermophilicity of this enzyme over that of the parent TrX enzyme (Figure 15).

Similarly, mutation of Leu 105 to His (L105H) in TrX-HML xylanase to produce the TrX-HML-105H mutant xylanase exhibits increased enzymatic activity over the parent TrX-HML xylanase at 70 and 75° C (Figure 4).

The Leu to His mutation at position 105 (L105H) improves the thermophilicity of TrX-HML-105H in comparison to TrX-HML xylanase. The L105H mutation represents a change from Leu, which is a hydrophobic, branched-chain amino acid to His bearing a relatively bulky, polar imidazole side-chain. Without wishing to be bound by theory, the L105H mutation introduces a reasonably bulky, planar amino acid capable of hydrogen bonding with other amino acids in the same vicinity of the molecule, possibly enhancing the intramolecular packing of atoms in the enzyme and thereby stabilizing the tertiary structure of the enzyme. Further, the imidazole side-chain may be protonated in the assay conditions to give the conjugate acid of imidazole. The protonated imidazole moiety may partake in attractive electrostatic interactions within the three dimensional tertiary structure of the xylanase and thereby stabilize its tertiary structure.

The combined mutant xylanase, TrX-HML-75A-105H, exhibited a maximum enzymatic activity at a temperature of 70° C and further showed greater enzymatic activity than either TrX-HML-75A or TrX-HML-105H single mutant xylanases at 70° C (Figure 4). These results indicate that the effects of the S75A and L105H mutations, on the thermophilicity of the mutant xylanase, are additive or complementary.

A series of TrX-HML xylanases bearing mutations at position-105 were constructed to determine those amino acid residues which enhance the thermophilicity

of the parent TrX-HML enzyme (Figure 5). Three mutants at position 105, TrX-HML-105H, TrX-HML-105R and TrX-HML-105K, showed greater enzymatic activity than the precursor TrX-HML enzyme from about 60° C or higher. The native xylanase comprises a Leu at position 105, a relatively hydrophobic branched-chain amino acid. Mutant xylanases wherein position 105 is substituted with a hydrophilic, positively charged or basic amino acid, for example His, Arg or Lys exhibited enhanced thermophilicity.

The combination mutant TrX-HML-75A-105R xylanase showed a similar temperature-activity profile to TrX-HML-75A-105H xylanase, suggesting that the S75A and L105R mutations, like those of the S75A and L105H mutations are additive or complementary. These results further suggest that basic residues at position 105 enhance the thermophilicity of the xylanases.

Due to the observed increase in thermophilicity associated with mutations involving position 75, combination mutants were also examined involving different substitutions at position 75, along with L105R. Three genetically modified xylanase mutants, TrX-HML-75C-105R, TrX-HML-75A-105R and TrX-HML-75G-105R showed greater enzymatic activity than either the precursor TrX-HML-105R xylanase or the TrX-HML xylanase at temperatures greater than about 60° C (Figure 6). A fourth mutant TrX-HML-75T-105R xylanase showed no enhancement in thermophilicity over the precursor TrX-HML-105R xylanase that has a natural Ser residue at position 75. The mutant threonine residue at position 75, like the natural Ser 75 residue found in TrX and TrX-HML parent xylanases, is a hydrophilic amino acid. Collectively, the mutations which involve replacing Ser at position 75 with small, nonpolar amino acids, such as but not wishing to be limiting Ala, Gly or Cys lead to an increase in the thermophilicity of the xylanase.

A series of mutant xylanases were also constructed with mutations Gln-125 to Ala and Ile-129 to Glu. The new mutants showed an increase of enzymatic activity at higher temperatures, as compared to their precursor xylanases (see Figures 7 to 9). These include (see Table 2 for complete description of modified enzymes):

- TrX-HML-125A;
- TrX-HML-125A129E;
- TrX-HML-GRAE;
- TrX-HML-AHAE;
- TrX-HML-GHAE;
- TrX-HML-ARAE;
- TrX-HML-GPHAE; and
- TrX-HML-GPRAE;

In some organisms, the expression and recovery of these modified xylanases may be reduced or not possible due to the synthesis of sites within the protein that reduce expression or recovery of the modified xylanase. This reduced recovery may vary depending upon the host within which the modified enzyme is expressed. For example, which is not to be construed as limiting, alterations of the amino acid sequence may produce a proteolytic cleavage site that is recognized by a protease in certain, but not all hosts. In order to overcome this problem adjacent amino acids, on one or both sides of the site comprising the desired mutation, may be modified in order to attend to any host-specific difficulty for the expression and recovery of a modified xylanase. Preferably, the additional amino acids that are altered do not negate the effect of the initially substituted amino acid in increasing the thermophilicity, or alkalophilicity, or both the thermophilicity and alkalophilicity, of the enzyme. For example, which is not to be considered limiting in any manner, a modified xylanase comprising a substitution of L105R, can be produced from *E.coli*, however, the recovery of this enzyme is reduced in *Trichoderma*, and *Aspergillus* due to endogenous KEX protease activity recognizing the amino acid combination "Lys-Arg" at positions 104 and 105 respectively. In this case, the amino acid at position 104 may be substituted for by an alternate amino acid, for example a non-polar amino acid as in modified xylanses TrX-HML-GPHAE, or TrX-HML-GPRAE. As shown in Figure 14, the substitution of Lys at positon 104 by Pro does not affect the thermophilicity or alkalophilicity of these modified xylanase. It is to be understood that other proteases may recognize other amino acid combinations, that may be produced when preparing the modified xylanases as described herein. Therefore, the

present invention also pertains to a modified xylanase comprising one or more substituted amino acids adjacent to the amino acids as described herein.

This invention therefore includes a modified xylanase comprising a His at position 10, a Met at position 27, a Leu at position 29, and at least one of:

- a non-polar amino acid at positions 75, 104, or 125, or a combination thereof;
- a polar amino acid at position 105; and
- an acidic amino acid at position 129.

Preferably, the amino acid at postion 75 is Ala, the amino acid at position 125 is selected from the group consisting of Ala, Cys, Gly, and Thr, the amino acid at position125 is Glu. the amino acid at position 105 is selected from the group consisting of His, Lys, and Arg, and the amino acid residue at position 104 is Pro.

Increasing the Alkalophilicity of Xylanase

The effect of pH conditions on the enzymatic activity of single mutant TrX-75A xylanase is shown in Figure 10. At 55° C, the TrX-75A mutant xylanase displays an increase in activity above pH 5.5 when compared to the native TrX enzyme over the same pH range. A similar contribution to improved alkalophilicity by the substitution of Ser for Ala at position 75 (Trx-75A) was also observed for the TrX-HML-75A over the parent TrX-HML xylanase at pH conditions between 6.5 and 7 (Figure 11).

An increase in alkalophilicity, with an increase in activity over pH from about 5.2 to a pH of about 6.5 is also observed in TrX-157D-161R-162H-165H, when compared with that of the native TrX over the same pH range (Figure 16).

The L105H mutation in the TrX-HML-105H mutant xylanase also increased the enzymatic activity over the parent TrX-HML xylanase at pH 6.5 and 7.0 (Figure 11).

Interestingly, the combination mutant TrX-HML-75A-105H xylanase showed greater enzymatic activity than either TrX-HML-75A or TrX-HML-105H single mutant xylanases at pH 6.5 and 7.0 (Figure 11), suggesting that the effects of the S75A mutation and the L105H mutation on the alkalophilicity of the xylanase are additive or complementary.

A series of genetically modified xylanases modified at position 105 were constructed to determine those residues which promote increased alkalophilicity in modified xylanases (Figure 12). Three mutant xylanases bearing three mutations at position 75, TrX-HML-105H, TrX-HML-105R and TrX-HML-105K showed greater enzymatic activity than the precursor TrX-HML xylanase at pH conditions of 6.5 and 7.0. Collectively, the mutations which lead to increases in alkalophilicity, represent a change from a branched chain relatively hydrophobic Leu residue to a residue which is hydrophilic, positively charged or basic.

Without wishing to be bound by theory, the hydrophilic, positively charged, or basic residues may facilitate intramolecular packing with other atoms that are juxtapositioned in the same vicinity in the tertiary structure of the xylanase. These residues may stabilize the three dimensional structure of the enzyme against structural perturbations in the molecule which may arise via the titration of several ionizable side-chains of amino aids in other regions of the molecule. Again, without wishing to be bound by theory, the basic ionized form of the side chain may be important in altering the pH activity profile of the enzyme, as at pH conditions between 6 and 7, Arg and Lys residues have side-chains which likely remain protonated. In contrast, His residues having a pKa of approximately 6 in solution for its imidazole moiety could be present in either a protonated or unprotonated form. However, it is known to those skilled in the art that the polarity of the substituents surrounding an amino acid side chain may affect its pKa value. For example, the side chain of a His residue in a polar or hydrophobic region of a protein may exhibit a pKa of 6 whereas the same side-chain in a hydrophobic or apolar environment may exhibit a pKa of 7 or greater.

In another study, mutations were constructed at position 75 of TrX-HML to determine which residues promote increased alkalophilicity in modified xylanases (Figure 13). Four xylanases bearing mutations at position 75, TrX-HML-75C-105R, TrX-HML-75A-105R, TrX-HML-75G-105R and TrX-HML-75T-105R showed greater enzymatic activity at pH conditions of 6.0, 6.5 and 7.0, compared to the precursors TrX-HML and TrX-HML-105R xylanases.

The mutations Q125A and I29E that enhanced the thermophilicity of xylanases, are compatible to the mutations at positions 75 and 105 described above, as the combination mutants like TrX-HML-75G-105H-125A129E possessing these two mutations generally maintained the pH/activity profile of the precursor xylanase TrX-HML-75G-105H (Figure 14).

A series of mutant xylanases were also constructed with mutations Gln-125 to Ala, and Ile-129 to Glu. The new mutants showed an increase of enzymatic activity at higher pH, as compared to their precursor xylanases (see Figures 11 to 14 and 17-19). These include (see Table 2 for complete description of modified enzymes):

- TrX-HML-125A;
- TrX-HML-125A129E;
- TrX-HML-GRAE;
- TrX-HML-AHAE;
- TrX-HML-GHAE;
- TrX-HML-ARAE;
- TrX-HML-GPHAE;
- TrX-HML-GPRAE;
- TrX-HML-AHAE-RR;
- TrX-HML-AHAE-RRR;
- TrX-HML-AHAE-RRR-DRHH;
- TrX-HML-AHA-RR-DRHH; and
- TrX-HML-AHAE-RR-DRHH

Mutant xylanases comprising a basic amino acid at positions 132 and 135, in addition to the substitutions describe above, including HML-75A-105H-125A-129E, exhibited an increase in alkalophilicity. Similarly, a mutant comprising a basic amino acid at positions 132, 135 and 144. also exhibited an increase in alkalophilicity. Examples of modified xylanase comprising these mutations include TrX-HML-AHAE-RR, and TrX-HML-AHAE-RRR (Figure 17).

Further modifications were made to xylanases in order to increase the alkalophilicity of the enzyme. For example, the substitution of an acidic amino acid at position 157, basic amino acid at positions 161, 162 and 165 with or without basic amino acid substitutions at positions 132, 135 and 144 also increased alkalophilicity. For example, TrX-HML-AHAE-RR-DRHH, or TrX-HML-AHAE-RRR-DRRH each exhibited an increase in alkalophilicity (Figures 18, 19) and a MEP of about 7.0 (Figure 20), when compared with TrX-HML-AHAE, which comprises a MEP of about 6.5, or TrX, with a MEP of about 5.6.

A further increase in alkalophilicity over those outlined above was also obtained by substitution of an acidic amino acid at position 157, and substituting a basic amino acid at positions 135, 144, 161, 162, 165, and leaving the amino acid at positions 129 and 132 in their native state, for example, TrX-HML-AHA-RR-DRHH (Figures 18 and 19). This enzyme exhibits a MEP of about 7.4.

The breadth of the pH optimum for TrX-HML-AHAE is much broader when compared to the pH profile of TrX (e.g. see Figure 19). Several of the modified xylanases of the present invention exhibit a breadth in the pH optimum approaching that of the breadth of native TrX, however, the pH optimum of these modified xylanases is shifted, with an increase of about at least 1 pH unit (Figure 19) when compared to that of TrX. TrX exhibits 80% of its optimal activity from about pH 4.8 to about pH 5.6 (pH optimum at 80% activity over 0.8 pH units). TrX-HML-AHAE exhibits a much broader pH range where 80% of its optimal activity ranges from about pH 4.8 to about pH 6.5 (about 1.7 pH units). The range of 80% of optimal activity for TrX-HML-AHAE-RR and

TrX-HML-AHAE-RRR, is from about 5.4 to about 6.6 (about 1.2 pH units; Figure 17), for TrX-HML-AHAE-RRR-DRHH and TrX-HML-AHAE-RR-DRHH is from about pH 5.8 to about 7.0 (about 1.2 pH units), and for TrX-HML-AHA-RR-DRHH is from about 5.9 to about 7.4 (about 1.5 pH units; see Figures 18 and 19).

Therefore, this invention also pertains to a modified xylanase comprising a His at position 10, a Met at position 27, a Leu at position 29, and at least one of:

- a non-polar amino acid at position 75, 104, or 125 or a combination thereof;
- a polar amino acid at position 105;
- an acidic amino acid at positions 129 and 157; and
- a basic amino acid at positions 132, 135, 144, 161, 162, or 165, or a combination thereof.

Preferably, the amino acid at position 75 is Ala, the amino acid at position 125 is selected from the group consisting of Ala, Cys, Gly, and Thr, the amino acid at position125 is Glu. The amino acid at position 105 is selected from the group consisting of His, Lys, and Arg, the amino acid residue at position 104 is Pro, the amino acid at position 132, 135, 144 and 161 is Arg, the amino acid at position 157 is Asp, and the amino acid at position 162 and 165 is His.

In summary, improved alkalophilic mutant TrX xylanases may be constructed through:

i) mutation of Ser 75 to a small non-polar residue, for example, but not limited to Ala. Furthermore, position 75 may be substituted by polar residues, for example, but not limited to Gly, Cys and Thr;

ii) mutation of Leu 105 to a basic residue such as but not limited to Arg, Lys or His;

iii) mutation of Gln 125 to Ala;

iv) mutation of Ile 129 to Glu;

v) mutation of Ala 132, Tyr 135, His 144, Gln 161, Gln 162, Thr 165 or a combination thereof to a basic amino acid, for example, Arg, Lys or His;

vi) mutation of Asn157 to an acidic amino acid, for example, Asp or Glu;

vii) combination of mutations described in i) with those described in ii) to iii) for the improvement of thermophilicity and alkalophilicity; or

viii) combination of mutations described in i) to vi), above, with the HML series of mutations as described above (see also U.S. 5,759,840).

The above description is not intended to limit the claimed invention in any manner, furthermore, the discussed combination of features might not be absolutely necessary for the inventive solution.

Examples

The present invention will be further illustrated in the following examples. However it is to be understood that these examples are for illustrative purposes only, and should not be used to limit the scope of the present invention in any manner.

EXAMPLE 1: Construction of *Trichoderma reesei* mutant xylanases

Basic recombinant DNA methods like plasmid preparation, restriction enzyme digestion, polymerase chain reaction, oligonucleotide phosphorylation, ligation, transformation and DNA hybridization were performed according to well-established protocols familiar to those skilled in the art (e.g. Sung et al., 1986) or as recommended by the manufacturer of the enzymes or kit. The buffers for many enzymes have been supplied as part of a kit or made according to the manufacturer's instructions. Restriction enzymes, T4 polynucleotide kinase and T4 DNA ligase were purchased from New England BioLabs Ltd, Mississauga, Ont. GeneAmp PCR reagent kit was purchased from Perkin-Elmer. A precursor plasmid pXYbc, which is a pUC type plasmid with a *Bacillus circulans* xylanase gene inserted, has previously been prepared and published (Sung et al, 1993; Campbell et al., U.S. Pat. No. 5,405,769). A

commonly used *E. coli* strain, HB101 (Clonetech Lab, Palo Alto, CA) was used as a transformation and expression host for all gene constructs. Birchwood xylan and Remazol Brilliant Blue R-D-Xylan were purchased from Sigma (St. Louis, Mo). Hydroxybenzoic acid hydrazide (HBAH) was purchased from Aldrich. Oligonucleotides were prepared with an APPLIED BIOSYSTEM DNA synthesizer (model 380B). All xylanase enzymatic assays were performed in a covered circulating water bath (Haake type F 4391) and maintained within a temperature range of $\pm$ 0.1° C.

1-1: Construction of precursor plasmid pTrX harbouring synthetic TrX (SEQ ID NO:39)

The precursor plasmid pTrX for mutations disclosed below has been previously published (Sung et al, 1995). This plasmid is derived from a pUC119 plasmid with a synthetic nucleotide sequence encoding a *Trichoderma reesei* xylanase (TrX; Figure 2). Expression of this xylanase and other mutant xylanases subsequently described are under the control of the *lac Z* promoter of the pUC plasmid. The total assembly of the *Trichoderma* xylanase gene required two stages, initially for the (92-190; Tr2 numbering) region, then followed by the (1-92; Tr2 numbering) region. The protocol for the construction of this gene is routine and identical to the standard published procedure for many other genes. The protocol requires enzymatic phosphorylation of overlapping synthetic oligonucleotides which encodes a xylanase. This is followed by their ligation into an appropriately cut plasmid.

For the construction of TrX (92-190), ten overlapping oligonucleotides (see Figure 2):

XyTv-101,	SEQ ID NO:29;
XyTv-102,	SEQ ID NO:30;
TrX-103,	SEQ ID NO:31;
XyTv-104,	SEQ ID NO:32;

XyTv-105,	SEQ ID NO:33;
XyTv-106,	SEQ ID NO:38;
XyTv-107,	SEQ ID NO:37;
TrX-108,	SEQ ID NO:36;
XyTv-109,	SEQ ID NO:35; and
XyTv-110,	SEQ ID NO:34

were designed with codon usage frequency imitating that of *E. coli*. The SalI and BglII cohesive ends of two terminal oligonucleotides enabled the enzymatic ligation of the ten fragments into the linearized plasmid pXYbc. The ten oligonucleotides (50 pmol, 1 μL for each) encoding the TrX(92-190) region of *Trichoderma* xylanase were phosphorylated in a mixture containing 10X standard kinase buffer (0.4 μL), 1 mM ATP (4 μL), T4 DNA kinase (5 units), and water (3 μL). Phosphorylation reactions were carried out for 1 h at 37° C. The solutions were then combined and heated to 70° C for 10 min. After being cooled slowly to room temperature, the combined solutions were added to a mixture of 4 mM ATP (3.5 μL), EcoR1-HindIII linearized plasmid pUC119 (0.1 pmol), and T4 DNA ligase (3.5 μL) and incubated at 12° C for 20 h. Aliquots of the ligation mixture were used to transform *E. coli* HB101 on YT plates (8 g yeast extract, 5 g bacto-tryptone, 5 g NaCl, 15 g of agar in 1 L of water) containing ampicillin (100 mg/L).

For the preparation of a hybridization probe, one of the oligonucleotides, for example XyTv-110 (10 pmol, 1 μL) was phosphorylated with ^{32}P-ATP (10 pmol, 3 μL) using T4 DNA kinase (1 μL), 10X kinase buffer (1 μL), and water (4 μL) at 37° C for 1 h.

Transformants were selected randomly for hybridization analysis. Colonies were grown on YT plates with ampicillin overnight, and transferred onto nylon filters. They were then denatured with 0.5N NaOH - 1.5M NaCl (10 min) and neutralized with 0.5N Tris-HCl (pH 7.0) - 1.5M NaCl (10 min). After ultraviolet irradiation at 254 nm for 8 min, the filters were washed with 6X SSC - 0.05% Triton X-100 for 30

min. Cell debris was scraped off completely. After another 30 min. in fresh solution, duplicate filters were transferred individually into separate mixtures of 6X SSC - 1% dextran sulphate - 0.05% TritonX-100 - 1X Denhardt's hybridization fluid. The ^{32}P-labelled probe was added to the filter. After 16 h at 45° C, the filter was washed twice with 6X SSC - 0.05% TritonX-100 at room temperature for 5 min. and then at 65° C for 30 min. Positively hybridized clones with the intermediate plasmid pBcX-TrX were identified by auto-radiographic analysis.

The above protocol, involving enzymatic phosphorylation of synthetic overlapping oligonucleotides and ligation into a linearized plasmid, was employed in the assembly of the TrX(1-92) region and in the cassette mutagenesis for the subsequent generation of other mutant xylanases described in this invention.

For the assembly of the TrX(1-92; Tr2 numbering) region to complete the full-length *Trichoderma reesei* xylanse II gene (TrX), the intermediate plasmid pBcX-TrX was linearized by NheI and KpnI endonucleases to release the DNA insert for BcX(1-83). With NheI and KpnI cohesive ends, eight overlapping oligonucleotides:

TrX-1,	SEQ ID NO:21;
XyTv-2,	SEQ ID NO:22;
TrX-3,	SEQ ID NO:23;
XyTv-4,	SEQ ID NO:24;
XyTv-5,	SEQ ID NO:28;
TrX-6,	SEQ ID NO:27;
XyTv-7,	SEQ ID NO:26; and
TrX-8	SEQ ID NO:25

encoding the TrX(1-91) sequence were ligated into the linearized plasmid pBcX-TrX (Figure 2), via the protocol described above. The new plasmid pTrX therefore harbored a synthetic TrX gene (SEQ ID NO:39).

All mutant xylanase genes described below have been constructed via the method of cassette mutagenesis. The protocol for cassette mutagenesis was identical to that described for gene assembly described above. Generally, cassette mutagenesis involved (i) enzymatic phosphorylation of overlapping synthetic oligonucleotides, (ii) ligation of synthetic oligonucleotides with a linearized plasmid, (iii) transformation of the plasmid into *E. coli* HB101 competent cells, (iv) identification of mutant transformants via hybridization with the labelled oligonucleotide, and (v) confirmation of the mutation through dideoxy nucleotide sequencing.

1-2: Construction of the precursor plasmid pTrX-HML

The construction of this precursor plasmid pTrX-HML has been described in detail in U.S. Pat. No. 5,759,840 (see Example 1N, herein incorporated by reference; plasmid termed pNI-TX13). TrX-HML comprises the native TrX xylanase, along with three mutations at N10H (Asn at position 10 is replaced with His), Y27M and N29L. The first thirty amino acids of the sequence comprising N10H, Y27M and N29L are shown below.

```
TrX                                  1   2   3   4   5   6   7   8
amino acid                           Q   T   I   Q   P   G   T   G
5'-CT AGC TAA GGA GG CTG CAG ATG CAA ACA ATA CAA CCA GGA ACC GGT
   3'-G ATT CCT CC GAC GTC TAC GTT TGT TAT GTT GGT CCT TGG  CCA
    NheI                                                   PinAI

  9  10  11  12  13  14  15  16  17  18  19  20  21  22  23  24
  Y   H   N   G   Y   F   Y   S   Y   W   N   D   G   H   G   G
TAC CAC AAC GGT TAC TTT TAC AGC TAT TGG AAC GAT GGC CAT GGA GGC
ATG GTG TTG CCA ATG AAA ATG TCG ATA ACC TTG CTA CCG GTA CCT CCG

 25  26  27  28  29  30
  V   T   M   T   L   G
GTC ACA ATG ACT CTG GGG
CAG TGT TAC TGA GAC CCC
```

1-3: Construction of the deletion plasmid pTrX(1-113)

Plasmid pTrX(1-113) comprises nucleotides 1-113 of SEQ ID NO:39 (nucleotides 1-113 of TrX) and cannot express an active xylanse. Such transformants are confirmed by the absence of a clearing zone or halo around the transformant colonies on blue xylan plates.

The new plasmid pTrX(1-113) was constructed via (i) the removal of the TrX(114-190) coding sequence of pTrX through cutting with restriction enzymes BamHI and BglII, (ii) ligation of the identical cohesive ends of the linearized plasmid, (iii) transformation into the *E. coli* HB101 competent cells followed by platting on YT plate (containing 5 g yeast extract, 3 g bacto-tryptone, 5 g NaCl, 15 g of agar in 1 L of water, 1 g Remazol Brilliant Blue R-D-xylan) and ampicillin (100 mg/L), (iv) identification of the mutant transformants through the loss of xylanase activity (absence of a clearing zone or halo around the colonies on the blue xylan plate overnight at 40°C), and (v) confirmation of the mutation through dideoxy nucleotide sequencing. The protocol for each of these steps was similar to that for gene assembly described above.

1-4: Construction of the deletion plasmid pTrX-HML(1-113)

Plasmid pTrX-HML(1-113) is similar to pTrX(1-113), but contains three mutations at positions 10, 27 and 29 (Tr2 numbering) of N10H, Y27M and N29L (as described above). The plasmid was constructed with the same protocol as described for pTrX(1-113; see above), in that the sequence encoding the TrX(114-190) region was deleted. The pTrX-HML(1-113) plasmid does not express an active xylanse.

1-5: Construction of pTrX-75A and pTrX-105H

All of the following mutant xylanase genes, based on the pTrX-derived plasmids pTrX(1-113) (see Example 1-3) and pTrX-HML(1-113) (see Example 1-4), were constructed using cassette mutagenesis. PCR primers that harbor specific mutations, were used to create PCR products. These PCR products were used to

complete the C-terminal sequence (residues 114-190; Tr2 numbering) of the full length xylanase genes. Appearance of clearing zones or halos around transformant colonies plated on plates containing blue xylan indicated that these colonies expressed an active xylanse and thus provides a marker for clones expressing a functional mutant TrX enzyme.

The protocol for the construction of these plasmids is similar to the protocol previously described for gene assembly (above). The procedure involved:

i) PCR with primer oligonucleotides bearing specific mutations at position -75 (in the case of pTrX-75A), or position -105 (in the case of pTrX-105H),

ii) cutting the PCR product with restriction enzymes HindIII at one end and KasI or EcoRI at the other,

iii) ligation of the restriction fragments to the HindIII/KasI- or EcoRI-linearized deletion plasmid,

iv) transformation into *E. coli* HB101 competent cells,

v) identification of mutant transformants expressing xylanase activity (indicated by the appearance of a clearing zone or halo surrounding colonies plated on media containing blue xylan), and

vi) confirmation of the mutation through dideoxy nucleotide sequencing.

The two xylanse mutants TrX-75A and TrX-105H comprise the sequence of TrX, with the exception of that the Ser at position 75 was replaced with an Ala residue (S75A) in TrX-75A, and the Leu at position 105 was replaced with a His residue (L105H) in TrX-105H.

The PCR primers used to create these genetically modified xylanses (specific mutation os shown in bold) include:

PCR oligonucleotide primers:

TX-75A-1 (SEQ ID NO:40)

```
  69  70  71  72  73  74  75  76  77  78  79  80   81
   N   G   N   S   Y   L   A   V   Y   G   W   S   R
5'-T GGG AAT TCA TAC TTA GCC GTC TAT GGC TGG TCT AG
      EcoRI
```

TX-105H-1 (SEQ ID NO:41)

```
100 101 102 103 104 105 106 107 108 109 110 111 112 113
  T   G   A   T   K   H   G   E   V   T   S   D   G   S  5'-ACC
GGC GCC ACA AAA CAC GGC GAA GTC ACT AGT GAT GGA TCC
      KasI
```

Reverse PCR primer TX-C1 comprised:

TX-C1 (SEQ ID NO:42)

```
183 184 185 186 187 188 189 190  ter
  G   S   A   S   I   T   V   S
CCA AGG CGA TCA TAA TGT CAC TCG ATT TCT AGA ACT TCG AAC CC-5'
                                    BglI     HindIII
```

The appropriate PCR template, oligonucleotide primers, and restriction enzymes to cut the end of the PCR products are listed below in Table 3-1.

Table 3-1

PCR product	PCR upstream primer	PCR reverse primer	PCR template	Restriction enzymes for PCR product
(a)	TX-75A-1	TX-C1	pTrX	EcoRI/ HindIII
(b)	TX-105H-1	TX-C1	pTrX	KasI/ HindIII

For the preparation of PCR product (a), plasmid pTrX was used as a template for PCR. The reaction solution contained plasmid pTrX DNA (50 ng, 15 μL), 5 μL 10X buffer (100 mM KCl, 100 mM ammonium sulfate, 200 mM Tris-HCl pH 8.8, 40 mM magnesium sulfate, 1% TritonX-100, 100 mg/ml BSA), 5 μL 5 mM dNTPs, PCR

primer TX-75A (25 pmol, 2.5 μL), and reverse PCR primer TX-C1 (25 pmol, 2.5 μL) and water (19 μL).

The reaction was covered with paraffin oil (50 μL) to prevent evaporation. The reaction mixture was pre-warmed to 94° C without enzyme for 5 min, then the reaction mixture was cooled to 72° C. Subsequently, DNA polymerase (1 μL,1U) was added to the reaction mixture. The reaction mixture was incubated in a temperature cycler for 30 cycles of 94° C for 1 min., 55°C for 2 min. and then 72°C for 2 min. The yield of the PCR product was approximately 1 μg of a 400 bp fragment. This fragment was purified from an agarose gel.

The EcoRI/HindIII-linearized PCR product (a) (Table 3-1) was ligated to the EcoRI/HindIII-linearized pTrX plasmid to generate plasmid pTrX-75A comprising full length xylanase with Ser at position 75 replaced with Ala (S75A).

In the same manner, PCR product (b) (Table 3-1) was prepared and was linearized with the KasI and HindIII restriction nucleases. The linearized product (b) was ligated to the KasI/HindIII-linearized pTrX plasmid to generate plasmid pTrX-105H comprising full length xylanase with Leu at position 105 replaced with His (L105H).

1-6: Construction of pTrX-HML-105H, pTrX-HML-105K and pTrX-HML-105R

Three mutant xylanses TrX-HML-105H, pTrX-HML-105K and pTrX-HML-105R are similar to TrX-HML except that Leu at position 105 is replaced by His (L105H), Lys (L105K) and Arg (L105R), respectively. As indicated previously, the TrX-HML xylanse is similar to the TrX xylanse except that Asn at position 10 is replaced with His (N10H), Tyr at position 27 is replaced by Met (Y27M) and Asn at position 29 is replaced by Leu (N29L).

A similar PCR product (b) for the synthesis of pTrX-105H was used for the construction of pTrX-HML-105H. The PCR primers with mutation (in bold type) in the construction of pTrX-HML-105K and pTrX-HML-105R are shown below.

Mutation PCR oligonucleotide primers:

TX-105K-1 (SEQ ID NO:43)

```
   100 101 102 103 104 105 106 107 108 109 110 111 112 113
     T   G   A   T   K   K   G   E   V   T   S   D   G   S
5'-ACC GGC GCC ACA AAA AAA GGC GAA GTC ACT AGT GAT GGA TCC
       KasI
```

TX-105R-1 (SEQ ID NO:44)

```
   100 101 102 103 104 105 106 107 108 109 110 111 112 113
     T   G   A   T   K   R   G   E   V   T   S   D   G   S
5'-ACC GGC GCC ACA AAA AGA GGC GAA GTC ACT AGT GAT GGA TCC
       KasI
```

The appropriate PCR template and primers, and the restriction enzymes to cut the end of the PCR products are listed below (Table 3-2).

Table 3-2

PCR product	PCR upstream primer	PCR reverse primer	PCR template	Restriction enzymes for PCR product
(c)	TX-105K-1	TX-C1	pTrX	KasI/ HindIII
(d)	TX-105R-1	TX-C1	pTrX	KasI/ HindIII

The PCR products (b) (Table 3-1) , (c) and (d) (Table 3-2) were prepared and cut with KasI and HindIII restriction nucleases. The products of the restriction digests (b), (c) and (d) were ligated into a KasI/HindIII-linearized pTrX-HML(1-113) plasmid to generate plasmids pTrX-HML-105H, pTrX-HML-105K and pTrX-HML-105R, respectively.

1-7: Construction of the plasmids pTrX-HML-75A and pTrX-HML-75A-105H

The two mutant xylanses TrX-HML-75A and TrX-HML-75A-105H are similar to TrX-HML except that Ser at position 75 is replaced by Ala (S75A) in TrX-HML-75A construct, and in TrX-HML-75A-105H Ser at position 75 is replaced by Ala (S75A) and Leu at position 105 is replaced by His (L105H).

The appropriate PCR template and primers, and the restriction enzymes to cut the end of the PCR products are listed below (Table 3-3).

Table 3-3

PCR product	PCR upstream primer	PCR reverse primer	PCR template	Restriction enzymes for PCR product
(e)	TX-75A-1	TX-C1	pTrX-105H	EcoRI/ HindIII

The EcoRI/HindIII-cut PCR products (a) and (e) (Tables 3-1 and 3-3 respectively) were prepared and ligated into KasI/HindIII-linearized pTrX-HML(1-113) plasmid to generate plasmids pTrX-HML-75A and pTrX-HML-75A-105H respectively.

1-8: Construction of pTrX-HML-75A-105R, pTrX-HML-75C-105R, pTrX-HML-75G-105R and pTrX-HML-75T-105R

Xylnase mutants TrX-HML-75A-105R, TrX-HML-75C-105R, TrX-HML-75G-105R and TrX-HML-75T-105R are similar to TrX-HML-105R (comprising mutations N10H, Y27M, N29L and L105R), with the exception of an additional single mutation S75A , S75C, S75G and S75T in each of the mutant xylanses, respectively.

The PCR primers with mutations S75C (TX-75C-1; SEQ ID NO:45), S75G (TX75-G-1; SEQ ID NO:46) and S75T (TX-75-T-1; seq id no:47) are shown below.

Mutation PCR oligonucleotide primers:

TX-75C-1 (SEQ ID NO:45)

```
  69  70  71  72  73  74  75  76  77  78  79  80  81
   N   G   N   S   Y   L   C   V   Y   G   W   S   R
5'-T GGG AAT TCA TAC TTA TGC GTC TAT GGC TGG TCT AG
      EcoRI
```

TX-75G-1 (SEQ ID NO:46)

```
  69  70  71  72  73  74  75  76  77  78  79  80  81
   N   G   N   S   Y   L   G   V   Y   G   W   S   R
5'-T GGG AAT TCA TAC TTA GGC GTC TAT GGC TGG TCT AG
          EcoRI
```

TX-75T-1 (SEQ ID NO:47)

```
  69  70  71  72  73  74  75  76  77  78  79  80  81
   N   G   N   S   Y   L   T   V   Y   G   W   S   R
5'-T GGG AAT TCA TAC TTA ACC GTC TAT GGC TGG TCT AG
      EcoRI
```

The appropriate PCR template and primers, and the restriction enzymes to cut the end of the PCR products are listed below (Table 3-4).

Table 3-4

PCR product	PCR upstream primer	PCR reverse primer	PCR template	Restriction enzymes for PCR product
(f)	TX-75A-1	TX-C1	pTrX-HML-105R	EcoRI/ HindIII
(g)	TX-75C-1	TX-C1	pTrX-HML-105R	EcoRI/ HindIII
(h)	TX-75G-1	TX-C1	pTrX-HML-105R	EcoRI/ HindIII
(i)	TX-75T-1	TX-C1	pTrX-HML-105R	EcoRI/ HindIII

The EcoRI/HindIII-cut PCR products (f), (g), (h) and (i) (see Table 3-4) were prepared and ligated into EcoRI/HindIII-linearized pTrX-HML(1-113) plasmid to generate plasmids pTrX-HML-75A-105R, pTrX-HML-75C-105R, pTrX-HML-75G-105R, and pTrX-HML-75T-105R respectively.

1.9:. Construction of the plasmids pTrX-HML-125A and pTrX-HML-125A129E.

The mutants TrX-HML-125A and TrX-HML-125A129E were identical to TrX-HML, with the exception of additional mutations Q125A and I129E.

The intact mutant genes were assembled via the ligation of two DNA sequences encoding the 1-121 and the 122-190 regions. The DNA sequence encoding the 1-121 region was created via deletion of the plasmid pTrX-HML by nucleases NheI and MluI. The DNA sequence encoding the 122-190 region was generated via PCR. The PCR primers with mutation Q125A or Q125A/I129E (in bold type) are shown below.

TX-125A-1 (SEQ ID NO:48)

```
 120 121 122 123 124 125 126 127 128 129 130 131 132 133
       Q   R   V   N   A   P   S   I   I   G   T   A   T
5'-C CAA CGC GTT AAT GCG CCA TCG ATC ATT GGA ACC GCC ACC
      MluI
```

TX-125A129E-1 (SEQ ID NO:49)

```
 120 121 122 123 124 125 126 127 128 129 130 131 132 133
       Q   R   V   N   A   P   S   I   E   G   T   A   T
5'-C CAA CGC GTT AAT GCG CCA TCG ATC GAG GGA ACC GCC ACC
      MluI
```

The appropriate PCR template and primers, and the restriction enzymes to cut the end of the PCR product which is the 122-190 sequence, are listed below (Table 3-5).

Table 3-5

PCR product	PCR upstream primer	PCR reverse primer	PCR template	Restriction enzymes for PCR product
(j)	TX-125A-1	TX-C1	pTrX	MluI/ HindIII
(k)	TX-125A129E-1	TX-C1	pTrX	MluI/ HindIII

The two cut DNA sequences 1-121 and 122-190 together constituting an intact xylanase sequence, were ligated to the NheI/HindIII-linearized plasmid pTrX-(1-113) to generate plasmids pTrX-HML-125A and pTrX-HML-125A129E.

1.10: Construction of the plasmid pTrX-HML-75G-105R-125A129E.

The mutant TrX-HML-75G-105R-125A129E was identical to TrX-HML-75G-105R, with the exception of the additional mutations Q125A and I129E.

The intact mutant genes were assembled via the ligation of two DNA sequences encoding the 1-121 and the 122-190 regions. The DNA sequecence encoding the 1-121 region prepared through the deletion of plasmid pTrX-HML-75G-105R with restriction nucleases listed below (Table 3-6).

Table 3-6

Deletion sequence	Precursor plasmid	Restriction enzymes for PCR product
(A)	pTrX-HML-75G-105R	NheI/ MluI

The DNA sequence encoding the 122-190 region was the same MluI/HindIII-cut PCR product (k) in the Example 1.9 (above).

The cut PCR product (k) and the deletion sequence (A) were ligated to the NheI/HindIII-linearized plasmid pTrX-(1-113) to generate the new plasmids listed below (Table 3-7).

Table 3-7

Deletion product	PCR product	New plasmid
(A)	(k)	pTrX-HML-75G-105R-125A129E

1.11: Construction of the plasmids pTrX-HML-75G-105H-125A129E, pTrX-HML-75A-105H-125A129E and pTrX-HML-75A-105R-125A129E.

The mutants TrX-HML-75G-105H-125A129E, pTrX-HML-75A-105H-125A129E and pTrX-HML-75A-105R-125A129E were identical to TrX-HML-75G-105R-125A129E, with the exception of the appropriate mutations at residues-75 (S75A or S75G) and -105 (L105H or L105R).

The intact mutant genes were assembled via the ligation of two DNA sequences encoding the 1-101 and the 102-190 regions.

For the preparation of the DNA sequence encoding the 1-101 region, restriction nucleases for the deletion of the appropriate plasmid are listed below (Table 3-8).

Table 3-8

Deletion sequence	Precursor plasmid	Restriction enzymes for PCR product
(B)	pTrX-HML-75G-105R	NheI/ KasI
(C)	pTrX-HML-75A-105R	NheI/ KasI

For the preparation of the DNA sequence encoding the 102-190 region, polymerase chain reaction was used. The appropriate PCR primers with mutations at position-105 and the restriction enzymes to cut the end of the PCR product are listed below (Table 3-9).

Table 3-9: Plasmid pTrX-HML-75G-105R-125A129E as PCR template.

PCR product	PCR upstream primer	PCR reverse primer	Restriction enzymes for PCR product
(l)	TX-105H-1	TX-C1	KasI/ HindIII
(m)	TX-105R-1	TX-C1	KasI/ HindIII

The cut PCR product ((l) or (m)) and one of the deletion sequences ((B) or (C)) were ligated to the NheI/HindIII-linearized plasmid pTrX-(1-113) to generate the new plasmids listed below (Table 3-10).

Table 3-10

Deletion product	PCR product	New plasmid
(B)	(l)	pTrX-HML-75G-105H-125A129E
(C)	(l)	pTrX-HML-75A-105H-125A129E
(C)	(m)	pTrX-HML-75A-105R-125A129E

1.12. Construction of the plasmids pTrX-HML-75G-104P105H-125A129E and pTrX-HML-75G-104P105R-125A129E.

The mutants TrX-HML-75G-104P105H-125A129E and pTrX-HML-75G-104P105R-125A129E were identical to TrX-HML-75G-105H-125A129E and TrX-HML-75G-105R-125A129E respectively, with the exception of an additional mutation of Lys-104 into proline (K104P).

The intact mutant genes were assembled via the ligation of two DNA sequences encoding the 1-101 and the 102-190 regions.

The DNA sequence encoding the 1-101 region for the three new mutants was the same deletion sequence (B) through the cutting of plasmid pTrX-HML-75G-105R by nucleases NheI and KasI in the Example 1.11 (above).

For the preparation of the DNA sequecence encoding the 102-190 region, polymerase chain reaction was used. The PCR primers with mutations at residues-104 and 105 (bold type) have been synthesized.

Mutation PCR oligonucleotide primers:

TX-104P-105H-1 (SEQ ID NO:50)

```
  100 101 102 103 104 105 106 107 108 109 110 111 112
    T   G   A   T   P   H   G   E   V   T   S   D
5'ACC GGC GCC ACA CCA CAC GGC GAA GTC ACT AGT GAT GG
      KasI
```

TX-104P-105R-1 (SEQ ID NO:51)

```
  100 101 102 103 104 105 106 107 108 109 110 111 112
    T   G   A   T   P   R   G   E   V   T   S   D
5'ACC GGC GCC ACA CCA AGA GGC GAA GTC ACT AGT GAT GG
      KasI
```

Polymerase chain reaction was conducted. The appropriate primers and restriction enzymes to cut the ends of the PCR product, were listed below (Table 1-11).

Table 1-11: Plasmid pTrX-HML-75G-105R-125A129E as the PCR template,

PCR product	PCR upstream primer	PCR reverse primer	Restriction enzymes for PCR product
(n)	TX-104P-105H-1	TX-C1	KasI/ HindIII
(o)	TX-104P-105R-1	TX-C1	KasI/ HindIII

The cut PCR product (n, or o) and the deletion sequence (B) were ligated to the NheI/HindIII-linearized plasmid pTrX-(1-113) to generate the new plasmids listed below (Table 3-12).

Table 3-12

Deletion product	PCR product	New Plasmid
(B)	(n)	pTrX-HML-75G-104P-105H-125A129E
(B)	(o)	pTrX-HML-75G-104P-105R-125A129E

1.13. Construction of the plasmids pTrX-157D-161R-162H-165H; pTrX-HML-75A-105H-125A-129E-132R-135R; pTrX-HML-75A-105H-125A-129E-132R-135R-144R; pTrX-HML-75A-105H-125A-129E-132R-135R-144R-157D-161R-162H-165H; pTrX-HML-75A-105H-125A-135R-144R-157D-161R-162H-165H;and pTrX-HML-75A-105H-125A-129E-135R-144R-157D-161R-162H-165H

The mutants: pTrX-157D-161R-162H-165H;
pTrX-HML-75A-105H-125A-129E-132R-135R;
pTrX-HML-75A-105H-125A-129E-132R-135R-144R;
pTrX-HML-75A-105H-125A-129E-132R-135R-144R-157D-161R-162H-165H;

pTrX-HML-75A-105H-125A-135R-144R-157D-161R-162H-165H; and pTrX-HML-75A-105H-125A-129E-135R-157D-144R161R-162H-165H, were prepared essentially as described above using the appropriate primers and templates. The intact mutant genes were assembled via the ligation of two DNA sequences encoding the 1-101 and the 102-190 regions.

Example 2: Characterization of mutant xylanases

2-1: Production of xylanases

The culture conditions comprised a 5 ml culture of overnight innoculant in 2YT medium (16 g bacto-tryptone, 10 g yeast extract, 5 g NaCl, 1 L of water) containing ampicillin (100 mg/L) was added to 2YT medium (1 L) with ampicillin. The cultures were grown with shaking (200 rpm) at 37°C. After 16 hr, cells were harvested.

2-2: Purification of mutant xylanases

Protein samples were prepared from cells by first making an extract of the cells by grinding 10 g of the cell paste with 25 g of alumina powder. After grinding to smooth mixture, small amounts (5 mL) of ice cold buffer A (10mM sodium acetate, pH 5.5 for BcX mutants) or buffer B (10mM sodium acetate, pH 4.6 for TX mutants) were added and the mixture ground vigorously between additions. The alumina and cell debris were removed by centrifugation of the mixture at 8000 x g for 30 min.

Prior to column chromatography, the supernatant was adjusted to pH 4.6 by acetic acid and centrifuged to remove any precipitate. The subsequent method for column chromatography was identical for all mutant xylanses.

Following acidification and centrifugation, the xylanse sample was pumped onto a 50 ml bed volume, CM-sepharose fast flow, cation exchange column (Pharmacia Biotech, Uppsala), equilibrated in 10 mM sodium acetate (pH 4.6). The xylanse was eluted with a 250 ml linear gradient (0 to 0.6M NaCl in 10 mM sodium acetate , pH 4.6) at a flow rate of 1 ml/min. The xylanses elute at 150 to 200 ml of the gradient. Aliquots from the collected fractions are examined by SDS-PAGE, and those fractions having most of the xylanse present were pooled. The purified xylanse was quantified by spectrophotometry at 280 nm using an extinction coefficient between 54,600 - 53,400 $M^{-1,}$ for most mutant TrX xylanses. A typical purification from 10g of cells yielded 25 mg of xylanse.

2-3: Standard assay for the measurement of enzymatic activity

The quantitative assay determined the number of reducing sugar ends generated from soluble xylan. The substrate for this assay was the fraction of birchwood xylan which dissolved in water from a 5% suspension of birchwood xylan (Sigma Chemical Co.). After removing the insoluble fraction, the supernatant was freeze dried and stored in a dessicator. The measurement of specific activity was performed as follows: Reaction mixtures containing 100 µL of 30 mg/mL xylan previously diluted in assay buffer (50 mM sodium citrate, pH 5.5 or the pH optimum of the tested xylanase), 150 µL assay buffer, and 50 µL of enzyme diluted in assay buffer were incubated at 40° C. At various time intervals 50 µL portions were removed and the reaction stopped by diluting in 1 mL of 5 mM NaOH. The amount of reducing sugars was determined with the hydroxybenzoic acid hydrazide reagent (HBAH) (Lever, 1972, Analytical Biochem 47:273-279). A unit of enzyme activity was defined as that amount generating 1 µ mol reducing sugar in 1 minute at 40° C.

For comparison of the specific activities between mutant and native xylanses the specific activities of a mutant xylanse was converted to a relative activity. The relative activity is calculated as a percentage, by dividing the

specific activity of the mutant enzyme by the specific activity of the native xylanse.

Table 4: Relative activity of TrX and native xylanases at 40°C.

Xylanse	Relative activity (%)
native TrX	100*
TrX-105H	97
TrX-75A	95
TrX-HML-75A-105H	95
TrX-HML-75A-105R	93

* specific activity of native TrX xylanase determined to be 770 U/mg.

The results depicted in Table 4 indicate that the specific enzymatic activities of the mutant xylanses at 40° C have not been changed significantly as compared to the native xylanse.

Example 3: Thermophilicity of mutant xylanases

Thermophilicity was examined to test the effect of different temperatures on the enzymatic hydrolysis of soluble xylan by different mutant xylanases.

The assay procedure was similar to the standard assay with changes in the incubation temperature and time. The xylanases (15 µg/mL) and soluble xylan substrate, in 50 mM sodium citrate buffer of pH 5.5, were mixed and incubated in a circulating water bath at different temperatures. After a 30 min incubation, the amount of reducing sugars released from xylan was determined by HBAH analysis and was calculated as a relative activity, with the value at 40°C representing 100%.

The effect of temperature on the hydrolysis of xylan by TrX and TrX-75A xylanses is shown in Figure 3. The mutant TrX-75A xylanse bearing a single S75A mutation, showed greater enzymatic activity than the natural TrX xylanase at 50, 55, 60 and 65° C. Further, the S75A mutation in the TrX-HML-75A mutant xylanase exhibited greater enzymatic activity than the TrX-HML parent xylanase at 70° C and 75° C (Figure 4). These results suggest that the S75A mutation improves the thermophilicity of TrX and TrX-HML xylanases.

Mutation of Leu 105 to His (L105H) in TrX-HML xylanase to produce the TrX-HML-105H mutant xylanase also exhibited increased enzymatic activity over the parent TrX-HML xylanase at 70 and 75° C (Figure 4).

Noteworthy, the combination mutant TrX-HML-75A-105H xylanase exhibited a maximum enzymatic activity at a temperature of 70° C and further showed greater enzymatic activity than either TrX-HML-75A or TrX-HML-105H single mutant xylanases at 70° C (Figure 4). These results suggest the effects of the two mutations S75A and L105H on the thermophilicity of the mutant xylanase are additive or complementary.

Substitution of Asn at position 157 with Asp, Ala at position 161 with Arg (A161R), Gln at position 162 with His (Q162H), and Thr at position165 with His (T165H) to produce TrX-157D-161R-162H-165H was neutral with respect to, or resulted in a slight increase in, the thermophilicity of this enzyme over that of the parent TrX enzyme (Figure 15).

A series of TrX-HML xylanses bearing mutations at position-105 were constructed to determine those amino acid residues which enhance the thermophilicity of the parent TrX-HML enzyme (Figure 5). Three mutants at position 105, TrX-HML-105H, TrX-HML-105R and TrX-HML-105K showed greater enzymatic activity than the precursor TrX-HML enzyme at 70° C or higher. The three mutations involve substituting Leu at position 105, a relatively hydrophobic branched-chain amino acid with His, Arg and Lys, amino acid

residues that are hydrophilic or positively charged or basic. Such mutations enhanced the thermophilicity of the mutant xylanases.

The combination mutant TrX-HML-75A-105R xylanse showed a similar temperature-activity profile to TrX-HML-75A-105H xylanse, suggesting that the S75A and L105R mutations, like the effect of the S75A and L105H are additive or complementary. These results further suggest that basic residues at position 105 enhance the thermophilicity of the xylanases.

In another series of mutant xylanases, position-75 of TrX-HML-105R was mutated to determine those residues which exhibited enhanced thermophilicity (Figure 6). Three genetically modified xylanase mutants, TrX-HML-75C-105R, TrX-HML-75A-105R and TrX-HML-75G-105R showed greater enzymatic activity than either the precursor TrX-HML-105R xylanse or the TrX-HML xylanse at temperatures greater than 60° C. Interestingly, the fourth mutant TrX-HML-75T-105R xylanse showed no enhancement in thermophilicity over the precursor TrX-HML-105R xylanse that has a natural Ser residue at position 75. The mutant threonine residue at position 75, like the natural Ser 75 residue found in TrX and TrX-HML parent xylanses, is a hydrophilic amino acid. Collectively, the mutations which involve replacing Ser, a polar amino acid at position 75 with small, nonpolar amino acids, such as but not wishing to be limiting Ala, Gly or Cys lead to an increase in the thermophilicity of the xylanase.

In another series, a mutation of the residue Gln-125 of TrX-HML to Ala (Q125A) resulted in greater activity at higher activity at higher temperatures (Figure 7). A second mutation of Ile-129 to Glu (I129E) also resulted a modest improvement of the thermophilicity of the xylanase. The advantageous mutations at residues-75, 105, 125 and 129 were then combined together to yield a mutant TrX-HML-75G-105R-125A129E and it showed further improvement of activity at higher temperatures (Figure 7). Other combination mutants with mutations at residues-75 (S75A or S75G) and -105 (L105H or L105R) have also demonstrated the same improvement of activity at higher temperature (Figure 8).

In the final series, a mutation of Lys-104 to proline (K104P) also produced a xylanase with much improved thermophilicity similar to the advantageous mutations S75G, L105R or H, Q125A and I129E (Figure 9).

Example 4 Alkalophilicity of mutant xylanases

The alkalophilicity of genetically modified xylanases was examined to test the effect that different pH conditions had on the enzymatic hydrolysis of soluble xylan by mutant xylanases. The assay procedure was similar to the standard assay with changes in the incubation temperature and time. Aliquots of genetically modified xylanses (15 μg/mL) and soluble xylan substrate in 50 mM sodium citrate buffers which varied between pH 4-7 were incubated together at 65° C. Following 30 min incubations, the amount of reducing sugars released from the xylan substrate was determined by HBAH analysis and the enzymatic activity as a function of pH was calculated for a variety of mutant xylanases with the maximal activity taken as 100%.

The effect of pH conditions on the enzymatic activity of single mutant TrX-75A xylanase is shown in Figure 10. At 55° C, the TrX-75A mutant xylanase displayed maximum activity at a pH which was higher (pH 5.5) than the pH at which the native TrX enzyme exhibits maximum activity (pH 5.0). An increase in enzymatic activity was also exhibited by the mutant in comparison to the natural TrX xylanase at pH conditions of 6.0 and 6.5.

A similar contribution to improved alkalophilicity by the S75A in Trx-75A was also observed for the TrX-HML-75A over the parent TrX-HML xylanase at pH conditions between 6.5 and 7 (Figure 11).

The L105H mutation in the TrX-HML-105H mutant xylanase also increased the enzymatic activity over the parent TrX-HML xylanase at pH 6.5 and 7.0 (Figure 11). Interestingly, the combination mutant TrX-HML-75A-105H xylanase showed greater enzymatic activity than either TrX-HML-75A or TrX-

HML-105H single mutant xylanases at pH 6.5 and 7.0 (Figure 11), suggesting that the effects of the S75A mutation and the L105H mutation on the alkalophilicity of the xylanase are additive or complementary.

A series of genetically modified xylanases modified at position 105 were constructed to determine those residues which promote increased alkalophilicity in modified xylanses (Figure 12). Three mutant xylanases bearing three mutations at position 75, TrX-HML-105H, TrX-HML-105R and TrX-HML-105K showed greater enzymatic activity than the precursor TrX-HML xylanse at pH conditions of 6.5 and 7.0. Collectively, the mutations which lead to increases in alkalophilicity, represent a change from a branched chain relatively hydrophobic Leu residue to a residue which is hydrophilic, positively charged or basic.

Without wishing to be bound by theory, the hydrophilic, positively charged, or basic residues may facilitate intramolecular packing with other atoms that are juxtapositioned in the same vicinity in the tertiary structure of the xylanse. These residues may stabilize the three dimensional structure of the enzyme against structural perturbations in the molecule which may arise via the titration of several ionizable side-chains of amino aids in other regions of the molecule. Again, without wishing to be bound by theory, the basic ionized form of the side chain may be important in altering the pH activity profile of the enzyme,as at pH conditions between 6 and 7, Arg and Lys residues have side-chains which likely remain protonated. In contrast, His residues having a pKa of approximately 6 in solution for its imidazole moiety could be present in either a protonated or unprotonated form. However, it is known to those skilled in the art that the polarity of the substituents surrounding an amino acid side chain may affect its pKa value. For example, the side chain of a His residue in a polar or hydrophobic region of a protein may exhibit a pKa of 6 whereas the same side-chain in a hydrophobic or apolar environment may exhibit a pKa of 7 or greater.

In another study, mutations were constructed at position 75 of TrX-HML to determine which residues promote increased alkalophilicity in modified

xylanases (Figure 13). Four xylanases bearing mutations at position 75, TrX-HML-75C-105R, TrX-HML-75A-105R, TrX-HML-75G-105R and TrX-HML-75T-105R showed greater enzymatic activity at pH conditions of 6.0, 6.5 and 7.0, compared to the precursors TrX-HML and TrX-HML-105R xylanases.

The two mutations Q125A and I129E in the mutant xylanases TrX-HML-125A and TrX-HML-125A129, which successfully increased the thermophilicity of the enzymes, have generally not affected their activity at higher pH, as compared to TrX-HML. This specific improvement of thermophilicity but not the alkalophilicity of xylanase, was also demonstrated in a comparison of TrX-HML-75A-105H and the combination mutants TrX-HML-75A-105H-125A129E (Figure 14). This has also been observed in other mutants TrX-HML-75G-105H-125A129E (Figure 14), TrX-HML-75A-105R-125A129E and TrX-HML-75G-105R-125A129E.

The substitution of an acidic amino acid at position 157, and basic amino acids at positions 161, 162 and 165 with or without basic amino acid substitutions at positions 132, 135 and 144 also increased alkalophilicity. TrX-HML-AHAE-RR-DRHH, or TrX-HML-AHAE-RRR-DRRH (see full description of substituted amino acids in Table 2) each exhibited an increase in alkalophilicity (Figures 18 and 19). These enzymes are also characterized as exhibiting a MEP of about pH 7.0 (Figures 18 and 19).

A further increase in alkalophilicity over those outlined above was also obtained by substituting an acidic amino acid at position 157, and basic amino acids at positions 135, 144, 161, 162, 165, and leaving the amino acid at positions 129 and 132 in their native state, for example, TrX-HML-AHA-RR-DRHH (Figures 18 and 19). The MEP of TrX-HML-AHA-RR-DRHH is about pH 7.4 (Figures 18 and 19).

In summary, improved alkalophilic mutant TrX xylanases may be constructed through i) mutation of Ser 75 to small apolar residues. Without

wishing to be limiting these residues may comprise Gly, Ala, and Cys; ii) mutation of Ser 75 to Thr; iii) mutation of Leu 105 to a basic residue such as but not limited to Arg, Lys or His; iv)mutation of Ala 132, Tyr 135, His 144, Gln 161, Gln 162, Thr 165 or a combination thereof to a basic amino acid, for example but not limited to, Arg, Lys or His; v) mutation of Asn157 to an acidic amino acid Asp or Glu, or vi) combination of mutations described in i) or ii) with those described in iii) and iv) for the improvement of alkalophilicity.

While the present invention has described mutant xylanases which exhibit improved thermophilicity and alkalophilicity and the benefits associated with these enzymes in the production of paper pulp, these mutant xylanases may also be of use in other industrial processes, for example but not limited to the washing of precision devices and semiconductors. Further, by virtue their increased thermophilicity, and thermostability the mutant xylanses may be used in chemical processes that employ small quantities of denaturants or detergents or in the presence of solvents, for example but not limited to small amounts of apolar solvents such as but not limited to hexane, dioxanes, carbontetrachloride, benzene, ethers, chloroform, acetic acid and methylene chloride, and polar solvents such as but not limited to acetone, alcohols, dimethylformamide, acetonitrile, sulfolane, dimethylsulfoxide and water.

The present invention has been described with regard to preferred embodiments. However, it will be obvious to persons skilled in the art that a number of variations and modifications can be made without departing from the scope of the invention as described herein.

All references and citations are herein incorporated by reference

References

Arase, A., Yomo, T., Urabe, I., Hata, Y., Katsube, Y. and Okada, H. (1993) FEBS Lett. 316:123-127.

Casimir-Schenkel, J., Davis, S., Fiechter, a., Gysin, B., Murray, E., Perrolaz, J.-J. and Zimmermann, W. European Patent application no. 91810652.7, published on 04.03.92. Publication no. 0 473 545 A2.

Campbell, R. L., Rose, D. R., Sung, W. L., Yaguchi, M. and Wakarchuk, W. US patent no. 5,405,769 issued on Apr. 11, 1995.

Campbell, R. L., Rose, D. R., Sung, W. L., Yaguchi, M. and Wakarchuk, W. PCT publication no. WO 94/24270, published on 27.10.1994.

Fisk, R. S. and Simpson, C. (1993) in Stability and Stabilization of Enzymes, edited by W. J. J. van den Tweel, A. Harder and R. M. Buitelaar; published by Elsevier Science Publishers B. V. pp323-328.

Gruber, K., Klintschar, G., Hayn, M, Schlacher, A., Steiner, W. and Kratky, C. (1998) Biochemistry 37:13475-13485.

Irwin, D., Jung, E. D. and Wilson, D. B. (1994) Appl. Environ. Microbiol. 60:763-770.

Lee, S. L., Forsberg, C. W., and Rattray, J. B. (1987) Appl. Environ. Microbiol. 53:644-650.

Lüthi, E., Jasmat, N. B., and Bergquist, P. L. (1990) Appl. Environ. Microbiol. 56:2677-2683.

Mathrani, I. M. and Ahring, B. K. (1992) Appl. Microbiol. Biotechnol. 38:23-27.

Misset, O. (1993) in Stability and Stabilization of Enzymes, edited by W. J. J. van den Tweel, A. Harder and R. M. Buitelaar; published by Elsevier Science Publishers B. V. pp111-131.

Nissen A. M., Anker, L., Munk, N., and Lange, N. K. in Xylans and Xylanases, edited by J. Visser, G. Beldman, M. A. Kusters-van Someren and A. G. J. Voragen, published by Elsevier, Amsterdam, 1992. p325-337.

Sakka, K., Kojima Y., Kondo, T., Karita, S., Ohmiya, K. and Shimada, K. (1993) Biosci. Biotech. Biochem. 57:273-277.

Simpson, H. D., Haufler, U. R., and Daniel, R. M. (1991) Biochem. J. (1991) 277:413-417.

Sung. W. L. . Yao. F.-L., Zahab, D. M. and Narang, S. A. (1986) Proc. Natl. Acad. Sci. USA 83:561-565.

Sung, W. L., Luk, C. K., Zahab, D. M. and Wakarchuk, W. (1993) Protein Expression Purif. 4:200-206.

Sung, W. L., Luk, C. K., Chan, B., Wakarchuk, W., Yaguchi, M., Campbell, R., Willick, G., Ishikawa, K. and Zabab, D. M. (1995) Biochem. Cell. Biol. 73:253-259.

Sung, W. L., Yaguchi, M and Ishikawa, K. US patent #5,759,840, issued on Jun. 2, 1998.

Sung, W. L., Yaguchi, M and Ishikawa, K. US patent #5,866,408, issued on Feb. 2, 1999

Tolan, J. S. and Vega Canovas, R. (1992) Pulp & Paper Canada 93:116-119).

Wakarchuck W. W., Sung, W. L., Campbell, R. L., Cunningham, A., Watson, D. C. and Yaguchi, M. (1994) Protein Engineering 7:1379-1386.

Wilson, D. B., Jung, E. D., Changas, G. S., Irvin, D. C. PCT international publication on 11 may 1995. Publication No. WO 95/12668.

Winterhalter C. and Liebl, W. (1995) Appl. Environ. Bicrobiol. 61:1810-1815.

Zappe, H., Jones, W. A., and Woods, D. R. (1987) Appl.Microbiol. Biotechnol. 27:57-63.

Zappe, H., Jones, W. A., and Woods, D. R. (1990) Nucleic Acids Res. 18:2179.

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OF PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. A modified xylanase comprising at least one substituted amino acid residue at a position selected from the group consisting of amino acid 75, 104, 105, 125, 129, 132, 135, 144, 157, 161, 162,and 165, said position determined from sequence alignment of said modified xylanase with *Trichoderma reesei* xylanase II amino acid sequence defined in SEQ ID NO:16.

2 The modified xylanase of claim 1, wherein said modified xylanase exhibits improved thermophilicity, alkalophilicity, or a combination thereof, in comparison to a corresponding native xylanase.

3. The modified xylanase of claim 1, wherein said substituted amino acid is at position 75 and is selected from the group consisting of a non-polar and a polar amino acid.

4. The modified xylanase of claim 3, wherein said at least one substituted amino acid is selected from the group consisting of Ala, Cys, Gly, and Thr.

5. The modified xylanase of claim 4, wherein said modified xylanase is derived from a Family 11 xylanase.

6. The modified xylanase of claim 5, wherein said Family 11 xylanase is a *Trichoderma reesei* xylanase.

7. The modified xylanase of claim 4, further comprising a His at position 10, Met at position 27 and Leu at position 29 (HML).

8. The modified xylanase of claim 1 wherein said at least one substituted amino acid residue is a polar amino acid at position 105, said modified xylanase further comprising a His at position 10, Met at position 27, Leu at position 29.

9. The modified xylanase of claim 8, wherein said polar amino acid is selected from the group consisting of His, Lys, and Arg.

10 The modified xylanase of claim 9, wherein the xylanase is a Family 11 xylanase.

11. The modified xylanase of claim 10, wherein said Family 11 xylanase is a *Trichoderma reesei* xylanase.

12. The modified xylanase of claim 11, further comprising a second substituted amino acid residue at position 75, said second substituted amino acid is selected from the group consisting of a non-polar and a polar amino acid.

13. The modified xylanase of claim 12, wherein said second substituted amino acid is selected from the group consisting of Ala, Cys, Gly, and Thr.

14 The modified xylanase of claim 13, wherein the xylanase is a Family 11 xylanase.

15. The modified xylanase of claim 14, wherein said Family 11 xylanase is a *Trichoderma reesei* xylanase.

16. The modified xylanase of claim 1 wherein said at least one substituted amino acid residue is a polar amino acid at position 157, 161, 162 and 165, said modified xylanase further comprising a His at position 10, Met at position 27, Leu at position 29.

17. The modified xylanase of claim 16, wherein said polar amino acid at positions 161, 162, and 165 is selected from the group consisting of Arg, Lys and His, and said polar amino acid at position 157 is selected from the group consisting of Asp and Glu.

18 The modified xylanase of claim 17, wherein the xylanase is a Family 11 xylanase.

19. The modified xylanase of claim 18, wherein said Family 11 xylanase is a *Trichoderma reesei* xylanase.

20. The modified xylanase of claim 1 wherein said at least one substituted amino acid residue is a non-polar amino acid at position 125, said modified xylanase further comprising a His at position 10, Met at position 27, Leu at position 29.

21. The modified xylanase of claim 20, wherein said at least one substituted amino acid is Ala.

22. The modified xylanase of claim 21, wherein said modified xylanase is derived from a Family 11 xylanase.

23. The modified xylanase of claim 22, wherein said Family 11 xylanase is a *Trichoderma reesei* xylanase.

24. The modified xylanase of claim 20, further comprising a second substituted amino acid at position 129, said second substituted amino acid is an acidic amino acid.

25. The modified xylanase of claim 24, wherein said second substituted amino acid is Glu.

26. The modified xylanase of claim 24, further comprising a third substituted amino acid residue at position 75, said third substituted amino acid is a nonpolar amino acid, and a fourth substituted amino acid residue at position 105, said fourth amino acid is a polar amino acid.

27. The modified xylanase of claim 26, wherein said third substituted amino acid is selected from the group consisting of Ala, Cys, Gly, and Thr, and wherein said fourth substituted amino acid is selected from the group consisting of His, Lys, and Arg.

28. The modified xylanase of claim 26, further comprising a fifth substituted amino acid residue at position 104, said fifth substituted amino acid is a non polar amino acid.

29. The modified xylanase of claim 28, wherein said fifth substituted amino acid is a Pro.

30. The modified xylanase of claim 26, further comprising a fifth substituted amino acid residue at position 132, and a sixth substituted amino acid residue at position 135, said fifth and sixth substituted amino acid each being a polar amino acid.

31. The modified xylanase of claim 30, further comprising a seventh substituted amino acid residue at position 144, said seventh substituted amino acid is a polar amino acid.

32. The modified xylanase of claim 31, further comprising an eighth substituted amino acid residue at position 157, a ninth substituted amino acid residue at position 161, a tenth substituted amino aicd at position 162, and an eleventh substituted amino acid residue at position 165, each of said eighth, ninth, tenth and eleventh substituted amino acid is a polar amino acid.

33. The modified xylanase of claim 26, further comprising a fifth substituted amino acid residue at position 157, a sixth substituted amino acid at position 161, a seventh substituted amino acid at position 162, and an eighth substituted amino acid at position 165, each of said fifth, sixth seventh and eighth substituted amino acid is a polar amino acid.

34. A use of the modified xylanase in claim 1 in an industrial process.

35. The use as defined in claim 34 wherein said industrial process is a pulp manufacturing.

36. A modified xylanase comprising at least one substituted amino acid residue, wherein said modified xylanase is characterized as having a maximum effective temperature (MET) between about 69°C to about 78°C, and wherein said modified xylanase is a Family 11 xylanase obtained from a *Trichoderma* sp..

37. The modified xylanase of claim 36, wherein said MET is between about 70° to about 75°C

38. A modified xylanase comprising at least one substituted amino acid residue, wherein said modified xylanase is characterized as having a maximum effective pH (MEP) between about pH 5.8 to about pH 7.6, and wherein said modified xylanase is a Family 11 xylanase obtained from a *Trichoderma* sp..

39. The modified xylanase of claim 38, wherein said MEP is between about pH 6.5 to about pH 7.4.

40. The modified xylanase of claim 36, wherein said modified xylanase is further characterized as having a maximum effective pH (MEP) is between about pH 5.8 to about pH 7.6.

41. The modified xylanase of claim 37, wherein said modified xylanase is further characterized as having a maximum effective pH (MEP) is between about pH 6.5 to about pH 7.4.

42. A modified xylanase selected from the group consisting of:

TrX-75A

TrX-161R-162H-165H;

TrX-HML-75A;

TrX-HML-105H;

TrX-HML-105R;

TrX-HML-105K;

TrX-HML-75A-105H;

TrX-HML-75A-105R;

TrX-HML-75C-105R;

TrX-HML-75G-105R;

TrX-HML-75T-105R

TrX-HML-125A;

TrX-HML-125A-129E;

TrX-HML-75G-105R-125A-129E (TrX-HML-GRAE);

TrX-HML-75A-105H-125A-129E (TrX-HML-AHAE);

TrX-HML-75G-105H-125A-129E (TrX-HML-GHAE);

TrX-HML-75A-105R-125A-129E (TrX-HML-ARAE);

TrX-HML-75G-104P-105R-125A-129E (TrX-HML-GPRAE);

TrX-HML-75G-104P-105H-125A-129E (TrX-HML-GPHAE);

TrX-HML-AHAE-RR;

TrX-HML-AHAE-RRR;

TrX-HML-AHAE-RRR-DRHH;

TrX-HML-AHA-RR-DRHH; and

TrX-HML-AHAE-RR-DRHH

SEQUENCE LISTING

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         35                  40                  45

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     50                  55                  60

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 65                  70                  75                  80

Tyr Ile Val Glu Asp Tyr Gly Asp Tyr Asn Pro Cys Ser Ser Ala Thr
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Ala Ser Tyr Leu Ala Val Tyr Gly Trp Val Asn Tyr Pro Gln Ala Glu
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Tyr Tyr Ile Val Glu Asp Tyr Gly Asp Tyr Asn Pro Cys Ser Ser Ala
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Thr Asp Thr Arg Ile Asn Glu Pro Ser Ile Thr Gly Thr Ser Thr Phe
115 120 125

Thr Gln Tyr Phe Ser Val Arg Glu Ser Thr Arg Thr Ser Gly Thr Val
130 135 140

Thr Val Ala Asn His Phe Asn Phe Trp Ala His His Gly Phe His Asn
145 150 155 160

Ser Asp Phe Asn Tyr Gln Val Val Ala Val Glu Ala Trp Ser Gly Ala
165 170 175

Gly Ser Ala Ala Val Thr Ile Ser Ser
180 185

<210> 3
<211> 185
<212> PRT
<213> Bacillus circulans

<400> 3
Ala Ser Thr Asp Tyr Trp Gln Asn Trp Thr Asp Gly Gly Gly Ile Val
1 5 10 15

Asn Ala Val Asn Gly Ser Gly Gly Asn Tyr Ser Val Asn Trp Ser Asn
20 25 30

Thr Gly Asn Phe Val Val Gly Lys Gly Trp Thr Thr Gly Ser Pro Phe
35 40 45

Arg Thr Ile Asn Tyr Asn Ala Gly Val Trp Ala Pro Asn Gly Asn Gly
50 55 60

Tyr Leu Thr Leu Tyr Gly Trp Thr Arg Ser Pro Leu Ile Glu Tyr Tyr
65 70 75 80

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Val Val Asp Ser Trp Gly Thr Tyr Arg Pro Thr Gly Thr Tyr Lys Gly
                85                  90                  95

Thr Val Lys Ser Asp Gly Gly Thr Tyr Asp Ile Tyr Thr Thr Thr Arg
            100                 105                 110

Tyr Asn Ala Pro Ser Ile Asp Gly Asp Arg Thr Thr Phe Thr Gln Tyr
        115                 120                 125

Trp Ser Val Arg Gln Ser Lys Arg Pro Thr Gly Ser Asn Ala Thr Ile
    130                 135                 140

Thr Phe Thr Asn His Val Asn Ala Trp Lys Ser His Gly Met Asn Leu
145                 150                 155                 160

Gly Ser Asn Trp Ala Tyr Gln Val Met Ala Thr Glu Gly Tyr Gln Ser
                165                 170                 175

Ser Gly Ser Ser Asn Val Thr Val Trp
            180                 185


<210> 4
<211> 201
<212> PRT
<213> Bacillus pumilus

<400> 4
Arg Thr Ile Thr Asn Asn Glu Met Gly Asn His Ser Gly Tyr Asp Tyr
  1                 5                  10                  15

Glu Leu Trp Lys Asp Tyr Gly Asn Thr Ser Met Thr Leu Asn Asn Gly
            20                  25                  30

Gly Ala Phe Ser Ala Gly Trp Asn Asn Ile Gly Asn Ala Leu Phe Arg
        35                  40                  45

Lys Gly Lys Lys Phe Asp Ser Thr Arg Thr His His Gln Leu Gly Asn
    50                  55                  60

Ile Ser Ile Asn Tyr Asn Ala Ser Phe Asn Pro Ser Gly Asn Ser Tyr
65                  70                  75                  80

Leu Cys Val Tyr Gly Trp Thr Gln Ser Pro Leu Ala Glu Tyr Tyr Ile
                85                  90                  95

Val Asp Ser Trp Gly Thr Tyr Arg Pro Thr Gly Ala Tyr Lys Gly Ser
            100                 105                 110

Phe Tyr Ala Asp Gly Gly Thr Tyr Asp Ile Tyr Glu Thr Thr Arg Val
        115                 120                 125

Asn Gln Pro Ser Ile Ile Gly Ile Ala Thr Phe Lys Gln Tyr Trp Ser
    130                 135                 140

Val Arg Gln Thr Lys Arg Thr Ser Gly Thr Val Ser Val Ser Ala His
145                 150                 155                 160

Phe Arg Lys Trp Glu Ser Leu Gly Met Pro Met Gly Lys Met Tyr Glu
                165                 170                 175

Thr Ala Phe Thr Val Glu Gly Tyr Gln Ser Ser Gly Ser Ala Asn Val
            180                 185                 190
```

```
Met Thr Asn Gln Leu Phe Ile Gly Asn
        195                 200


<210> 5
<211> 185
<212> PRT
<213> Bacillus subtilis

<400> 5
Ala Ser Thr Asp Tyr Trp Gln Asn Trp Thr Asp Gly Gly Gly Ile Val
  1               5                  10                  15

Asn Ala Val Asn Gly Ser Gly Gly Asn Tyr Ser Val Asn Trp Ser Asn
             20                  25                  30

Thr Gly Asn Phe Val Val Gly Lys Gly Trp Thr Thr Gly Ser Pro Phe
         35                  40                  45

Arg Thr Ile Asn Tyr Asn Ala Gly Val Trp Ala Pro Asn Gly Asn Gly
     50                  55                  60

Tyr Leu Thr Leu Tyr Gly Trp Thr Arg Ser Pro Leu Ile Glu Tyr Tyr
 65                  70                  75                  80

Val Val Asp Ser Trp Gly Thr Tyr Arg Pro Thr Gly Thr Tyr Lys Gly
                 85                  90                  95

Thr Val Lys Ser Asp Gly Gly Thr Tyr Asp Ile Tyr Thr Thr Thr Arg
            100                 105                 110

Tyr Asn Ala Pro Ser Ile Asp Gly Asp Arg Thr Thr Phe Thr Gln Tyr
        115                 120                 125

Trp Ser Val Arg Gln Ser Lys Arg Pro Thr Gly Ser Asn Ala Thr Ile
    130                 135                 140

Thr Phe Ser Asn His Val Asn Ala Trp Lys Ser His Gly Met Asn Leu
145                 150                 155                 160

Gly Ser Asn Trp Ala Tyr Gln Val Met Ala Thr Glu Gly Tyr Gln Ser
                165                 170                 175

Ser Gly Ser Ser Asn Val Thr Val Trp
            180                 185


<210> 6
<211> 211
<212> PRT
<213> Clostridium acetobutylicum

<400> 6
Ser Ala Phe Asn Thr Gln Ala Ala Pro Lys Thr Ile Thr Ser Asn Glu
  1               5                  10                  15

Ile Gly Val Asn Gly Gly Tyr Asp Tyr Glu Leu Trp Lys Asp Tyr Gly
             20                  25                  30

Asn Thr Ser Met Thr Leu Lys Asn Gly Gly Ala Phe Ser Cys Gln Trp
         35                  40                  45

Ser Asn Ile Gly Asn Ala Leu Phe Arg Lys Gly Lys Lys Phe Asn Asp
```

```
     50                  55                  60

Thr Gln Thr Tyr Lys Gln Leu Gly Asn Ile Ser Val Asn Tyr Asn Cys
 65                  70                  75                  80

Asn Tyr Gln Pro Tyr Gly Asn Ser Tyr Leu Cys Val Tyr Gly Trp Thr
                 85                  90                  95

Ser Ser Pro Leu Val Glu Tyr Tyr Ile Val Asp Ser Trp Gly Ser Trp
            100                 105                 110

Arg Pro Pro Gly Gly Thr Ser Lys Gly Thr Ile Thr Val Asp Gly Gly
        115                 120                 125

Ile Tyr Asp Ile Tyr Glu Thr Thr Arg Ile Asn Gln Pro Ser Ile Gln
    130                 135                 140

Gly Asn Thr Thr Phe Lys Gln Tyr Trp Ser Val Arg Arg Thr Lys Arg
145                 150                 155                 160

Thr Ser Gly Thr Ile Ser Val Ser Lys His Phe Ala Ala Trp Glu Ser
                165                 170                 175

Lys Gly Met Pro Leu Gly Lys Met His Glu Thr Ala Phe Asn Ile Glu
            180                 185                 190

Gly Tyr Gln Ser Ser Gly Lys Ala Asp Val Asn Ser Met Ser Ile Asn
        195                 200                 205

Ile Gly Lys
    210


<210> 7
<211> 206
<212> PRT
<213> Clostridium stercorarium

<400> 7
Gly Arg Ile Ile Tyr Asp Asn Glu Thr Gly Thr His Gly Gly Tyr Asp
  1               5                  10                  15

Tyr Glu Leu Trp Lys Asp Tyr Gly Asn Thr Ile Met Glu Leu Asn Asp
             20                  25                  30

Gly Gly Thr Phe Ser Cys Gln Trp Ser Asn Ile Gly Asn Ala Leu Phe
         35                  40                  45

Arg Lys Gly Arg Lys Phe Asn Ser Asp Lys Thr Tyr Gln Glu Leu Gly
     50                  55                  60

Asp Ile Val Val Glu Tyr Gly Cys Asp Tyr Asn Pro Asn Gly Asn Ser
 65                  70                  75                  80

Tyr Leu Cys Val Tyr Gly Trp Thr Arg Asn Phe Leu Val Glu Tyr Tyr
                 85                  90                  95

Ile Val Glu Ser Trp Gly Ser Trp Arg Pro Pro Gly Ala Thr Pro Lys
            100                 105                 110

Gly Thr Ile Thr Gln Trp Met Ala Gly Thr Tyr Glu Ile Tyr Glu Thr
        115                 120                 125
```

```
Thr Arg Val Asn Gln Pro Ser Ile Asp Gly Thr Ala Thr Phe Gln Gln
    130                 135                 140

Tyr Trp Ser Val Arg Thr Ser Lys Arg Thr Ser Gly Thr Ile Ser Val
145                 150                 155                 160

Thr Glu His Phe Lys Gln Trp Glu Arg Met Gly Met Arg Met Gly Lys
                165                 170                 175

Met Tyr Glu Val Ala Leu Thr Val Glu Gly Tyr Gln Ser Ser Gly Tyr
            180                 185                 190

Ala Asn Val Tyr Lys Asn Glu Ile Arg Ile Gly Ala Asn Pro
        195                 200                 205
```

<210> 8
<211> 211
<212> PRT
<213> Ruminococcus flavefaciens

<400> 8

```
Ser Ala Ala Asp Gln Gln Thr Arg Gly Asn Val Gly Gly Tyr Asp Tyr
  1               5                  10                  15

Glu Met Trp Asn Gln Asn Gly Gln Gly Gln Ala Ser Met Asn Pro Gly
            20                  25                  30

Ala Gly Ser Phe Thr Cys Ser Trp Ser Asn Ile Glu Asn Phe Leu Ala
        35                  40                  45

Arg Met Gly Lys Asn Tyr Asp Ser Gln Lys Lys Asn Tyr Lys Ala Phe
    50                  55                  60

Gly Asn Ile Val Leu Thr Tyr Asp Val Glu Tyr Thr Pro Arg Gly Asn
65                  70                  75                  80

Ser Tyr Met Cys Val Tyr Gly Trp Thr Arg Asn Pro Leu Met Glu Tyr
                85                  90                  95

Tyr Ile Val Glu Gly Trp Gly Asp Trp Arg Pro Pro Gly Asn Asp Gly
            100                 105                 110

Glu Val Lys Gly Thr Val Ser Ala Asn Gly Asn Thr Tyr Asp Ile Arg
        115                 120                 125

Lys Thr Met Arg Tyr Asn Gln Pro Ser Leu Asp Gly Thr Ala Thr Phe
    130                 135                 140

Pro Gln Tyr Trp Ser Val Arg Gln Thr Ser Gly Ser Ala Asn Asn Gln
145                 150                 155                 160

Thr Asn Tyr Met Lys Gly Thr Ile Asp Val Ser Lys His Phe Asp Ala
                165                 170                 175

Trp Ser Ala Ala Gly Leu Asp Met Ser Gly Thr Leu Tyr Glu Val Ser
            180                 185                 190

Leu Asn Ile Glu Gly Tyr Arg Ser Asn Gly Ser Ala Asn Val Lys Ser
        195                 200                 205

Val Ser Val
    210
```

<210> 9
<211> 197
<212> PRT
<213> Schizophyllum commune

<400> 9
Ser Gly Thr Pro Ser Ser Thr Gly Thr Asp Gly Gly Tyr Tyr Tyr Ser
1 5 10 15

Trp Trp Thr Asp Gly Ala Gly Asp Ala Thr Tyr Gln Asn Asn Gly Gly
20 25 30

Gly Ser Tyr Thr Leu Thr Trp Ser Gly Asn Asn Gly Asn Leu Val Gly
35 40 45

Gly Lys Gly Trp Asn Pro Gly Ala Ala Ser Arg Ser Ile Ser Tyr Ser
50 55 60

Gly Thr Tyr Gln Pro Asn Gly Asn Ser Tyr Leu Ser Val Tyr Gly Trp
65 70 75 80

Thr Arg Ser Ser Leu Ile Glu Tyr Tyr Ile Val Glu Ser Tyr Gly Ser
85 90 95

Tyr Asp Pro Ser Ser Ala Ala Ser His Lys Gly Ser Val Thr Cys Asn
100 105 110

Gly Ala Thr Tyr Asp Ile Leu Ser Thr Trp Arg Tyr Asn Ala Pro Ser
115 120 125

Ile Asp Gly Thr Gln Thr Phe Glu Gln Phe Trp Ser Val Arg Asn Pro
130 135 140

Lys Lys Ala Pro Gly Gly Ser Ile Ser Gly Thr Val Asp Val Gln Cys
145 150 155 160

His Phe Asp Ala Trp Lys Gly Leu Gly Met Asn Leu Gly Ser Glu His
165 170 175

Asn Tyr Gln Ile Val Ala Thr Glu Gly Tyr Gln Ser Ser Gly Thr Ala
180 185 190

Thr Ile Thr Val Thr
195

<210> 10
<211> 191
<212> PRT
<213> Streptomyces lividans

<400> 10
Asp Thr Val Val Thr Thr Asn Gln Glu Gly Thr Asn Asn Gly Tyr Tyr
1 5 10 15

Tyr Ser Phe Trp Thr Asp Ser Gln Gly Thr Val Ser Met Asn Met Gly
20 25 30

Ser Gly Gly Gln Tyr Ser Thr Ser Trp Arg Asn Thr Gly Asn Phe Val
35 40 45

Ala Gly Lys Gly Trp Ala Asn Gly Gly Arg Arg Thr Val Gln Tyr Ser

```
     50                  55                  60

Gly Ser Phe Asn Pro Ser Gly Asn Ala Tyr Leu Ala Leu Tyr Gly Trp
 65                  70                  75                  80

Thr Ser Asn Pro Leu Val Glu Tyr Tyr Ile Val Asp Asn Trp Gly Thr
                85                  90                  95

Tyr Arg Pro Thr Gly Glu Tyr Lys Gly Thr Val Thr Ser Asp Gly Gly
            100                 105                 110

Thr Tyr Asp Ile Tyr Lys Thr Thr Arg Val Asn Lys Pro Ser Val Glu
        115                 120                 125

Gly Thr Arg Thr Phe Asp Gln Tyr Trp Ser Val Arg Gln Ser Lys Arg
    130                 135                 140

Thr Gly Gly Thr Ile Thr Thr Gly Asn His Phe Asp Ala Trp Ala Arg
145                 150                 155                 160

Ala Gly Met Pro Leu Gly Asn Phe Ser Tyr Tyr Met Ile Asn Ala Thr
                165                 170                 175

Glu Gly Tyr Gln Ser Ser Gly Thr Ser Ser Ile Asn Val Gly Gly
            180                 185                 190
```

<210> 11
<211> 191
<212> PRT
<213> Streptomyces lividans

<400> 11

```
Ala Thr Thr Ile Thr Thr Asn Gln Thr Gly Thr Asp Gly Met Tyr Tyr
  1               5                  10                  15

Ser Phe Trp Thr Asp Gly Gly Gly Ser Val Ser Met Thr Leu Asn Gly
            20                  25                  30

Gly Gly Ser Tyr Ser Thr Gln Trp Thr Asn Cys Gly Asn Phe Val Ala
        35                  40                  45

Gly Lys Gly Trp Ser Thr Gly Asp Gly Asn Val Arg Tyr Asn Gly Tyr
    50                  55                  60

Phe Asn Pro Val Gly Asn Gly Tyr Gly Cys Leu Tyr Gly Trp Thr Ser
65                  70                  75                  80

Asn Pro Leu Val Glu Tyr Tyr Ile Val Asp Asn Trp Gly Ser Tyr Arg
                85                  90                  95

Pro Thr Gly Thr Tyr Lys Gly Thr Val Ser Ser Asp Gly Gly Thr Tyr
            100                 105                 110

Asp Ile Tyr Gln Thr Thr Arg Tyr Asn Ala Pro Ser Val Glu Gly Thr
        115                 120                 125

Lys Thr Phe Gln Gln Tyr Trp Ser Val Arg Gln Ser Lys Val Thr Ser
    130                 135                 140

Gly Ser Gly Thr Ile Thr Thr Gly Asn His Phe Asp Ala Trp Ala Arg
145                 150                 155                 160
```

```
Ala Gly Met Asn Met Gly Gln Phe Arg Tyr Tyr Met Ile Asn Ala Thr
                165                 170                 175

Glu Gly Tyr Gln Ser Ser Gly Ser Ser Asn Ile Thr Val Ser Gly
            180                 185                 190


<210> 12
<211> 189
<212> PRT
<213> Streptomyces sp.

<400> 12
Ala Thr Thr Ile Thr Asn Glu Thr Gly Tyr Asp Gly Met Tyr Tyr Ser
  1               5                  10                  15

Phe Trp Thr Asp Gly Gly Gly Ser Val Ser Met Thr Leu Asn Gly Gly
             20                  25                  30

Gly Ser Tyr Ser Thr Arg Trp Thr Asn Cys Gly Asn Phe Val Ala Gly
         35                  40                  45

Lys Gly Trp Ala Asn Gly Gly Arg Arg Thr Val Arg Tyr Thr Gly Trp
     50                  55                  60

Phe Asn Pro Ser Gly Asn Gly Tyr Gly Cys Leu Tyr Gly Trp Thr Ser
 65                  70                  75                  80

Asn Pro Leu Val Glu Tyr Tyr Ile Val Asp Asn Trp Gly Ser Tyr Arg
                85                  90                  95

Pro Thr Gly Glu Thr Arg Gly Thr Val His Ser Asp Gly Gly Thr Tyr
            100                 105                 110

Asp Ile Tyr Lys Thr Thr Arg Tyr Asn Ala Pro Ser Val Glu Ala Pro
        115                 120                 125

Ala Ala Phe Asp Gln Tyr Trp Ser Val Arg Gln Ser Lys Val Thr Ser
    130                 135                 140

Gly Thr Ile Thr Thr Gly Asn His Phe Asp Ala Trp Ala Arg Ala Gly
145                 150                 155                 160

Met Asn Met Gly Asn Phe Arg Tyr Tyr Met Ile Asn Ala Thr Glu Gly
                165                 170                 175

Tyr Gln Ser Ser Gly Ser Ser Thr Ile Thr Val Ser Gly
            180                 185


<210> 13
<211> 189
<212> PRT
<213> Thermomonospora fusca

<400> 13
Ala Val Thr Ser Asn Glu Thr Gly Tyr His Asp Gly Tyr Phe Tyr Ser
  1               5                  10                  15

Phe Trp Thr Asp Ala Pro Gly Thr Val Ser Met Glu Leu Gly Pro Gly
             20                  25                  30

Gly Asn Tyr Ser Thr Ser Trp Arg Asn Thr Gly Asn Phe Val Ala Gly
```

```
         35                  40                  45

Lys Gly Trp Ala Thr Gly Gly Arg Arg Thr Val Thr Tyr Ser Ala Ser
     50                  55                  60

Phe Asn Pro Ser Gly Asn Ala Tyr Leu Thr Leu Tyr Gly Trp Thr Arg
 65                  70                  75                  80

Asn Pro Leu Val Glu Tyr Tyr Ile Val Glu Ser Trp Gly Thr Tyr Arg
                 85                  90                  95

Pro Thr Gly Thr Tyr Met Gly Thr Val Thr Thr Asp Gly Gly Thr Tyr
            100                 105                 110

Asp Ile Tyr Lys Thr Thr Arg Tyr Asn Ala Pro Ser Ile Glu Gly Thr
        115                 120                 125

Arg Thr Phe Asp Gln Tyr Trp Ser Val Arg Gln Ser Lys Arg Thr Ser
    130                 135                 140

Gly Thr Ile Thr Ala Gly Asn His Phe Asp Ala Trp Ala Arg His Gly
145                 150                 155                 160

Met His Leu Gly Thr His Asp Tyr Met Ile Met Ala Thr Glu Gly Tyr
                165                 170                 175

Gln Ser Ser Gly Ser Ser Asn Val Thr Leu Gly Thr Ser
            180                 185
```

<210> 14
<211> 190
<212> PRT
<213> Trichoderma harzianum

<400> 14

```
Gln Thr Ile Gly Pro Gly Thr Gly Tyr Ser Asn Gly Tyr Tyr Tyr Ser
  1               5                  10                  15

Tyr Trp Asn Asp Gly His Ala Gly Val Thr Tyr Thr Asn Gly Gly Gly
            20                  25                  30

Gly Ser Phe Thr Val Asn Trp Ser Asn Ser Gly Asn Phe Val Gly Gly
        35                  40                  45

Lys Gly Trp Gln Pro Gly Thr Lys Asn Lys Val Ile Asn Phe Ser Gly
     50                  55                  60

Ser Tyr Asn Pro Asn Gly Asn Ser Tyr Leu Ser Ile Tyr Gly Trp Ser
 65                  70                  75                  80

Arg Asn Pro Leu Ile Glu Tyr Tyr Ile Val Glu Asn Phe Gly Thr Tyr
                 85                  90                  95

Asn Pro Ser Thr Gly Ala Thr Lys Leu Gly Glu Val Thr Ser Asp Gly
            100                 105                 110

Ser Val Tyr Asp Ile Tyr Arg Thr Gln Arg Val Asn Gln Pro Ser Ile
        115                 120                 125

Ile Gly Thr Ala Thr Phe Tyr Gln Tyr Trp Ser Val Arg Arg Asn His
    130                 135                 140
```

```
Arg Ser Ser Gly Ser Val Asn Thr Ala Asn His Phe Asn Ala Trp Ala
145                 150                 155                 160

Ser His Gly Leu Thr Leu Gly Thr Met Asp Tyr Gln Ile Val Ala Val
                165                 170                 175

Glu Gly Tyr Phe Ser Ser Gly Ser Ala Ser Ile Thr Val Ser
            180                 185                 190


<210> 15
<211> 178
<212> PRT
<213> Trichoderma reesei

<400> 15
Ala Ser Ile Asn Tyr Asp Gln Asn Tyr Gln Thr Gly Gly Gln Val Ser
  1               5                  10                  15

Tyr Ser Pro Ser Asn Thr Gly Phe Ser Val Asn Trp Asn Thr Gln Asp
            20                  25                  30

Asp Phe Val Val Gly Val Gly Trp Thr Thr Gly Ser Ser Ala Pro Ile
        35                  40                  45

Asn Phe Gly Gly Ser Phe Ser Val Asn Ser Gly Thr Gly Leu Leu Ser
    50                  55                  60

Val Tyr Gly Trp Ser Thr Asn Pro Leu Val Glu Tyr Tyr Ile Met Glu
65                  70                  75                  80

Asp Asn His Asn Tyr Pro Ala Gln Gly Thr Val Lys Gly Thr Val Thr
                85                  90                  95

Ser Asp Gly Ala Thr Tyr Thr Ile Trp Glu Asn Thr Arg Val Asn Glu
            100                 105                 110

Pro Ser Ile Gln Gly Thr Ala Thr Phe Asn Gln Tyr Ile Ser Val Arg
        115                 120                 125

Asn Ser Pro Arg Thr Ser Gly Thr Val Thr Val Gln Asn His Phe Asn
    130                 135                 140

Trp Ala Ser Leu Gly Leu His Leu Gly Gln Met Met Asn Tyr Gln Val
145                 150                 155                 160

Val Ala Val Glu Gly Trp Gly Gly Ser Gly Ser Ala Ser Gln Ser Val
                165                 170                 175

Ser Asn


<210> 16
<211> 190
<212> PRT
<213> Trichoderma reesei

<400> 16
Gln Thr Ile Gln Pro Gly Thr Gly Tyr Asn Asn Gly Tyr Phe Tyr Ser
  1               5                  10                  15

Tyr Trp Asn Asp Gly His Gly Gly Val Thr Tyr Thr Asn Gly Pro Gly
```

```
            20                  25                  30

Gly Gln Phe Ser Val Asn Trp Ser Asn Ser Gly Asn Phe Val Gly Gly
        35                  40                  45

Lys Gly Trp Gln Pro Gly Thr Lys Asn Lys Val Ile Asn Phe Ser Gly
    50                  55                  60

Ser Tyr Asn Pro Asn Gly Asn Ser Tyr Leu Ser Val Tyr Gly Trp Ser
65                  70                  75                  80

Arg Asn Pro Leu Ile Glu Tyr Tyr Ile Val Glu Asn Phe Gly Thr Tyr
                85                  90                  95

Asn Pro Ser Thr Gly Ala Thr Lys Leu Gly Glu Val Thr Ser Asp Gly
            100                 105                 110

Ser Val Tyr Asp Ile Tyr Arg Thr Gln Arg Val Asn Gln Pro Ser Ile
        115                 120                 125

Ile Gly Thr Ala Thr Phe Tyr Gln Tyr Trp Ser Val Arg Arg Asn His
    130                 135                 140

Arg Ser Ser Gly Ser Val Asn Thr Ala Asn His Phe Asn Ala Trp Ala
145                 150                 155                 160

Gln Gln Gly Leu Thr Leu Gly Thr Met Asp Tyr Gln Ile Val Ala Val
                165                 170                 175

Glu Gly Tyr Phe Ser Ser Gly Ser Ala Ser Ile Thr Val Ser
            180                 185                 190


<210> 17
<211> 190
<212> PRT
<213> Trichoderma viride

<400> 17
Gln Thr Ile Gln Pro Gly Thr Gly Phe Asn Asn Gly Tyr Phe Tyr Ser
  1               5                  10                  15

Tyr Trp Asn Asp Gly His Gly Gly Val Thr Tyr Thr Asn Gly Pro Gly
            20                  25                  30

Gly Gln Phe Ser Val Asn Trp Ser Asn Ser Gly Asn Phe Val Gly Gly
        35                  40                  45

Lys Gly Trp Gln Pro Gly Thr Lys Asn Lys Val Ile Asn Phe Ser Gly
    50                  55                  60

Ser Tyr Asn Pro Asn Gly Asn Ser Tyr Leu Ser Val Tyr Gly Trp Ser
65                  70                  75                  80

Arg Asn Pro Leu Ile Glu Tyr Tyr Ile Val Glu Asn Phe Gly Thr Tyr
                85                  90                  95

Asn Pro Ser Thr Gly Ala Thr Lys Leu Gly Glu Val Thr Ser Asp Gly
            100                 105                 110

Ser Val Tyr Asp Ile Tyr Arg Thr Gln Arg Val Asn Gln Pro Ser Ile
        115                 120                 125
```

```
Ile Gly Thr Ala Thr Phe Tyr Gln Tyr Trp Ser Val Arg Arg Thr His
    130                 135                 140

Arg Ser Ser Gly Ser Val Asn Thr Ala Asn His Phe Asn Ala Trp Ala
145                 150                 155                 160

Gln Gln Gly Leu Thr Leu Gly Thr Met Asp Tyr Gln Ile Val Ala Val
                165                 170                 175

Glu Gly Tyr Phe Ser Ser Gly Ser Ala Ser Ile Thr Val Ser
            180                 185                 190


<210> 18
<211> 202
<212> PRT
<213> Fibrobacter succinogenes

<400> 18
Asn Ser Ser Val Thr Gly Asn Val Gly Ser Ser Pro Tyr His Tyr Glu
  1               5                  10                  15

Ile Trp Tyr Gln Gly Gly Asn Asn Ser Met Thr Phe Tyr Asp Asn Gly
             20                  25                  30

Thr Tyr Lys Ala Ser Trp Asn Gly Thr Asn Asp Phe Leu Ala Arg Val
         35                  40                  45

Gly Phe Lys Tyr Asp Glu Lys His Thr Tyr Glu Glu Leu Gly Pro Ile
     50                  55                  60

Asp Ala Tyr Tyr Lys Trp Ser Lys Gln Gly Ser Ala Gly Gly Tyr Asn
 65                  70                  75                  80

Tyr Ile Gly Ile Tyr Gly Trp Thr Val Asp Pro Leu Val Glu Tyr Tyr
                85                  90                  95

Ile Val Asp Asp Trp Phe Asn Lys Pro Gly Ala Asn Leu Leu Gly Gln
            100                 105                 110

Arg Lys Gly Glu Phe Thr Val Asp Gly Asp Thr Tyr Glu Ile Trp Gln
        115                 120                 125

Asn Thr Arg Val Gln Gln Pro Ser Ile Lys Gly Thr Gln Thr Phe Pro
    130                 135                 140

Gln Tyr Phe Ser Val Arg Lys Ser Ala Arg Ser Cys Gly His Ile Asp
145                 150                 155                 160

Ile Thr Ala His Met Lys Lys Trp Glu Glu Leu Gly Met Lys Met Gly
                165                 170                 175

Lys Met Tyr Glu Ala Lys Val Leu Val Glu Ala Gly Gly Gly Ser Gly
            180                 185                 190

Ser Phe Asp Val Thr Tyr Phe Lys Met Thr
        195                 200


<210> 19
<211> 189
<212> PRT
<213> Aspergillus awamorii
```

<400> 19
Arg Ser Thr Pro Ser Ser Thr Gly Glu Asn Asn Gly Tyr Tyr Tyr Ser
1 5 10 15

Phe Trp Thr Asp Gly Gly Gly Asp Val Thr Tyr Thr Asn Gly Asn Ala
20 25 30

Gly Ser Tyr Ser Val Glu Trp Ser Asn Val Gly Asn Phe Val Gly Gly
35 40 45

Lys Gly Trp Asn Pro Gly Ser Ala Lys Asp Ile Thr Tyr Ser Gly Asn
50 55 60

Phe Thr Pro Ser Gly Asn Gly Tyr Leu Ser Val Tyr Gly Trp Thr Thr
65 70 75 80

Asp Pro Leu Ile Glu Tyr Tyr Ile Val Glu Ser Tyr Gly Asp Tyr Asn
85 90 95

Pro Gly Ser Gly Gly Thr Thr Arg Gly Asn Val Ser Ser Asp Gly Ser
100 105 110

Val Tyr Asp Ile Tyr Thr Ala Thr Arg Thr Asn Ala Pro Ser Ile Asp
115 120 125

Gly Thr Gln Thr Phe Ser Gln Tyr Trp Ser Val Arg Gln Asn Lys Arg
130 135 140

Val Gly Gly Thr Val Thr Thr Ser Asn His Phe Asn Ala Trp Ala Lys
145 150 155 160

Leu Gly Met Asn Leu Gly Thr His Asn Tyr Gln Ile Leu Ala Thr Glu
165 170 175

Gly Tyr Gln Ser Ser Gly Ser Ser Ser Ile Thr Ile Gln
180 185

<210> 20
<211> 194
<212> PRT
<213> Thermomyces lanuginosus

<400> 20
Gln Thr Thr Pro Asn Ser Glu Gly Trp His Asp Gly Tyr Tyr Tyr Ser
1 5 10 15

Trp Trp Ser Asp Gly Gly Ala Gln Ala Thr Tyr Thr Asn Leu Glu Gly
20 25 30

Gly Thr Tyr Glu Ile Ser Trp Gly Asp Gly Gly Asn Leu Val Gly Gly
35 40 45

Lys Gly Trp Asn Pro Gly Leu Asn Ala Arg Ala Ile His Phe Glu Gly
50 55 60

Val Tyr Gln Pro Asn Gly Asn Ser Tyr Leu Ala Val Tyr Gly Trp Thr
65 70 75 80

Arg Asn Pro Leu Val Glu Tyr Tyr Ile Val Glu Asn Phe Gly Thr Tyr
85 90 95

Asp Pro Ser Ser Gly Ala Thr Asp Leu Gly Thr Val Glu Cys Asp Gly

100 105 110

Ser Ile Tyr Arg Leu Gly Lys Thr Thr Arg Val Asn Ala Pro Ser Ile
115 120 125

Asp Gly Thr Gln Thr Phe Asp Gln Tyr Trp Ser Val Arg Gln Asp Lys
130 135 140

Arg Thr Ser Gly Thr Val Gln Thr Gly Cys His Phe Asp Ala Trp Ala
145 150 155 160

Arg Ala Gly Leu Asn Val Asn Gly Asp His Tyr Tyr Gln Ile Val Ala
165 170 175

Thr Glu Gly Tyr Phe Ser Ser Gly Tyr Ala Arg Ile Thr Val Ala Asp
180 185 190

Val Gly

<210> 21
<211> 76
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: TrX-1

<400> 21
ctagctaagg aggctgcaga tgcaaacaat acaaccagga accggttaca acaacggtta 60

cttttacagc tattgg 76

<210> 22
<211> 78
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: XyTv-2

<400> 22
aacgatggcc atggtggtgt tacctataca aacgggcccg gaggccaatt tagcgtcaat 60

tggtctaact ccggaaac 78

<210> 23
<211> 78
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: TrX-3

<400> 23
ttcgtaggtg gaaaaggttg gcaacccggg accaaaaata aggtgatcaa cttctctgga 60

tcttataatc cgaatggg 78

<210> 24
<211> 74
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: XyTv-4

<400> 24
aattcatact taagcgtcta tggctggtct agaaacccac tgattgaata ttacattgtc 60

gaaaatttcg gtac 74

<210> 25
<211> 51
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: TrX-8

<400> 25
gattcctccg acgtctacgt ttgttatgtt ggtccttggc caatgttgtt g 51

<210> 26
<211> 84
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: XyTv-7

<400> 26
ccaatgaaaa tgtcgataac cttgctaccg gtaccaccac aatggatatg tttgcccggg 60

cctccggtta aatcgcagtt aacc 84

<210> 27
<211> 78
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: TrX-6

<400> 27
agattgaggc ctttgaagca tccacctttt ccaaccgttg ggccctggtt tttattccac 60

tagttgaaga gacctaga 78

<210> 28

<211> 85
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: XyTv-5

<400> 28
atattaggct tacccttaag tatgaattcg cagataccga ccagatcttt gggtgactaa 60

cttataatgt aacagctttt aaagc 85

<210> 29
<211> 58
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: XyTv-101

<400> 29
tcgacaattt cggtacctac aatccgagta ccggcgccac aaaattaggc gaagtcac 58

<210> 30
<211> 53
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: XyTv-102

<400> 30
tagtgatgga tccgtatatg atatctaccg tacccaacgc gttaatcagc cat 53

<210> 31
<211> 59
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: TrX-103

<400> 31
cgatcattgg aaccgccacc ttttatcagt actggagtgt tagacgtaat catcggagc 59

<210> 32
<211> 69
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: XyTv-104

<400> 32

tccggttcgg ttaatactgc gaatcacttt aatgcatggg cacagcaagg gttaacccta 60

ggtacaatg 69

<210> 33
<211> 67
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: XyTv-105

<400> 33
gattatcaaa tcgtagcggt ggaaggctac ttctcgagtg gttccgctag tattacagtg 60

agctaaa 67

<210> 34
<211> 73
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: XyTv-110

<400> 34
gttaaagcca tggatgttag gctcatggcc gcggtgtttt aatccgcttc agtgatcact 60

acctaggcat ata 73

<210> 35
<211> 54
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: XyTv-109

<400> 35
ctatagatgg catgggttgc gcaattagtc ggtagctagt aaccttggcg gtgg 54

<210> 36
<211> 60
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: XyTv-108

<400> 36
aaaatagtca tgacctcaca atctgcatta gtagcctcga ggccaagcca attatgacgc 60

<210> 37

<211> 66
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: XyTv-107

<400> 37
ttagtgaaat tacgtacccg tgtcgttccc aattgggatc catgttacct aatagtttag 60

catcgc 66

<210> 38
<211> 53
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: XyTv-106

<400> 38
caccttccga tgaagagctc accaaggcga tcataatgtc actcgatttc tag 53

<210> 39
<211> 596
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: TrX

<400> 39
ctagctaagg aggctgcaga tgcaaacaat acaaccagga accggttaca acaacggtta 60

cttttacagc tattggaacg atggccatgg tggtgttacc tatacaaacg ggcccggagg 120

ccaatttagc gtcaattggt ctaactccgg aaacttcgta ggtggaaaag gttggcaacc 180

cgggaccaaa aataaggtga tcaacttctc tggatcttat aatccgaatg ggaattcata 240

cttaagcgtc tatggctggt ctagaaaccc actgattgaa tattacattg tcgaaaattt 300

cggtacctac aatccgagta ccggcgccac aaaattaggc gaagtcacta gtgatggatc 360

cgtatatgat atctaccgta cccaacgcgt taatcagcca tcgatcattg gaaccgccac 420

cttttatcag tactggagtg ttagacgtaa tcatcggagc tccggttcgg ttaatactgc 480

gaatcacttt aatgcatggg cacagcaagg gttaacccta ggtacaatgg attatcaaat 540

cgtagcggtg gaaggctact tctcgagtgg ttccgctagt attacagtga gctaaa 596

<210> 40
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Tx-75a-1

<400> 40
tgggaattca tacttagccg tctatggctg gtctag 36

<210> 41
<211> 42
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Tx-105H-1

<400> 41
accggcgcca caaaacacgg cgaagtcact agtgatggat cc 42

<210> 42
<211> 44
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Tx-C1

<400> 42
ccaaggcgat cataatgtca ctcgatttct agaacttcga accc 44

<210> 43
<211> 42
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Tx-105K-1

<400> 43
accggcgcca caaaaaaagg cgaagtcact agtgatggat cc 42

<210> 44
<211> 42
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Tx-105R-1

<400> 44
accggcgcca caaaaagagg cgaagtcact agtgatggat cc 42

<210> 45
<211> 36
<212> DNA

<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Tx-75C-1

<400> 45
tgggaattca tacttatgcg tctatggctg gtctag 36

<210> 46
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Tx-75G-1

<400> 46
tgggaattca tacttaggcg tctatggctg gtctag 36

<210> 47
<211> 36
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Tx-75T-1

<400> 47
tgggaattca tacttaaccg tctatggctg gtctag 36

<210> 48
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: TX-125A-1

<400> 48
ccaacgcgtt aatgcgccat cgatcattgg aaccgccacc 40

<210> 49
<211> 40
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: TX-125A-129E-1

<400> 49
ccaacgcgtt aatgcgccat cgatcgaggg aaccgccacc 40

<210> 50

<211> 38
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: TX-104P-105H-1

<400> 50
accggcgcca caccacacgg cgaagtcact agtgatgg 38

<210> 51
<211> 38
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: TX-104P-105R-1

<400> 51
accggcgcca caccaagagg cgaagtcact agtgatgg 38

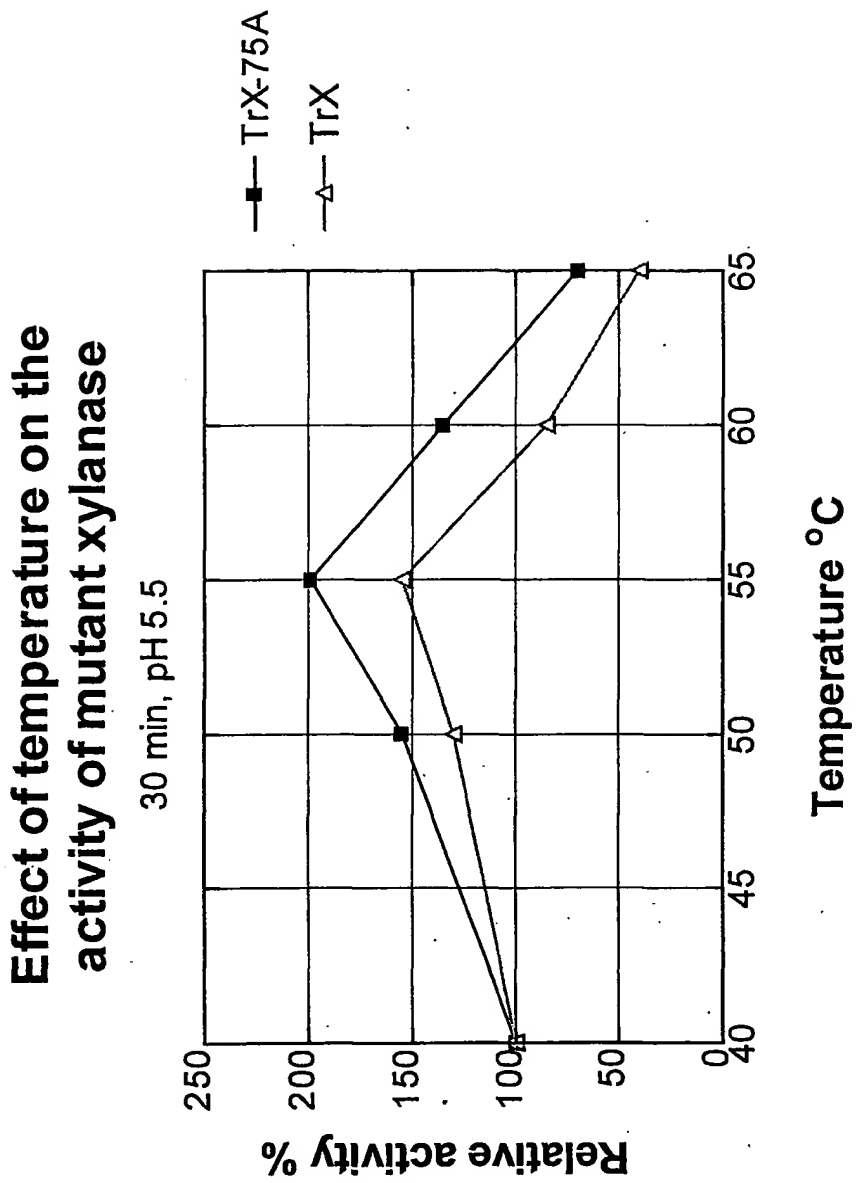
Effect of temperature on the activity of mutant xylanase
30 min, pH 5.5
TrX-75A
TrX
Relative activity %
250
200
150
100
50
0
40
45
50
55
60
65
Temperature °C

Figure 3

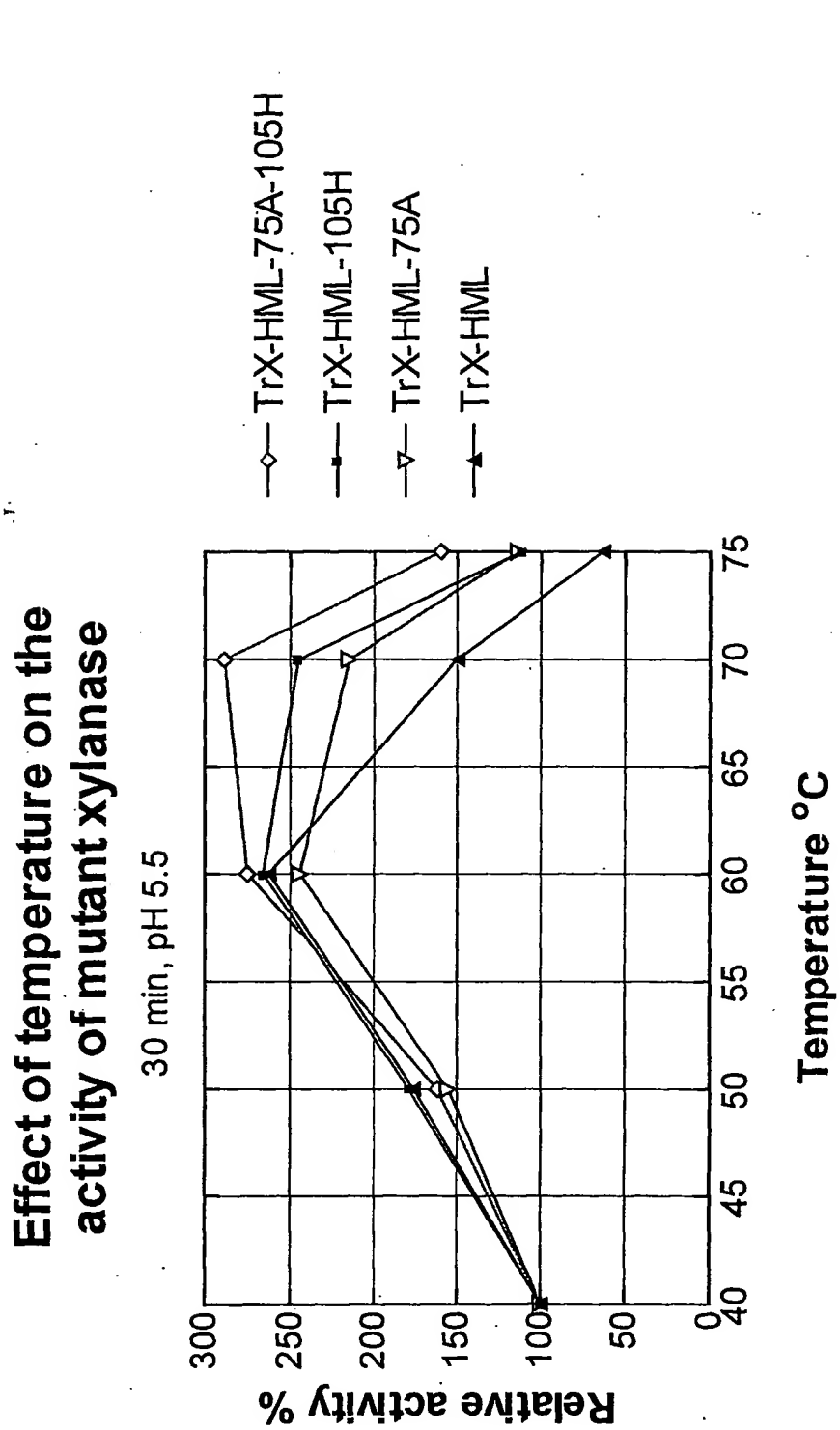
Effect of temperature on the activity of mutant xylanase
30 min, pH 5.5
TrX-HML-75A-105H
TrX-HML-105H
TrX-HML-75A
TrX-HML
Relative activity %
Temperature °C
300
250
200
150
100
50
0
40
45
50
55
60
65
70
75

Figure 4

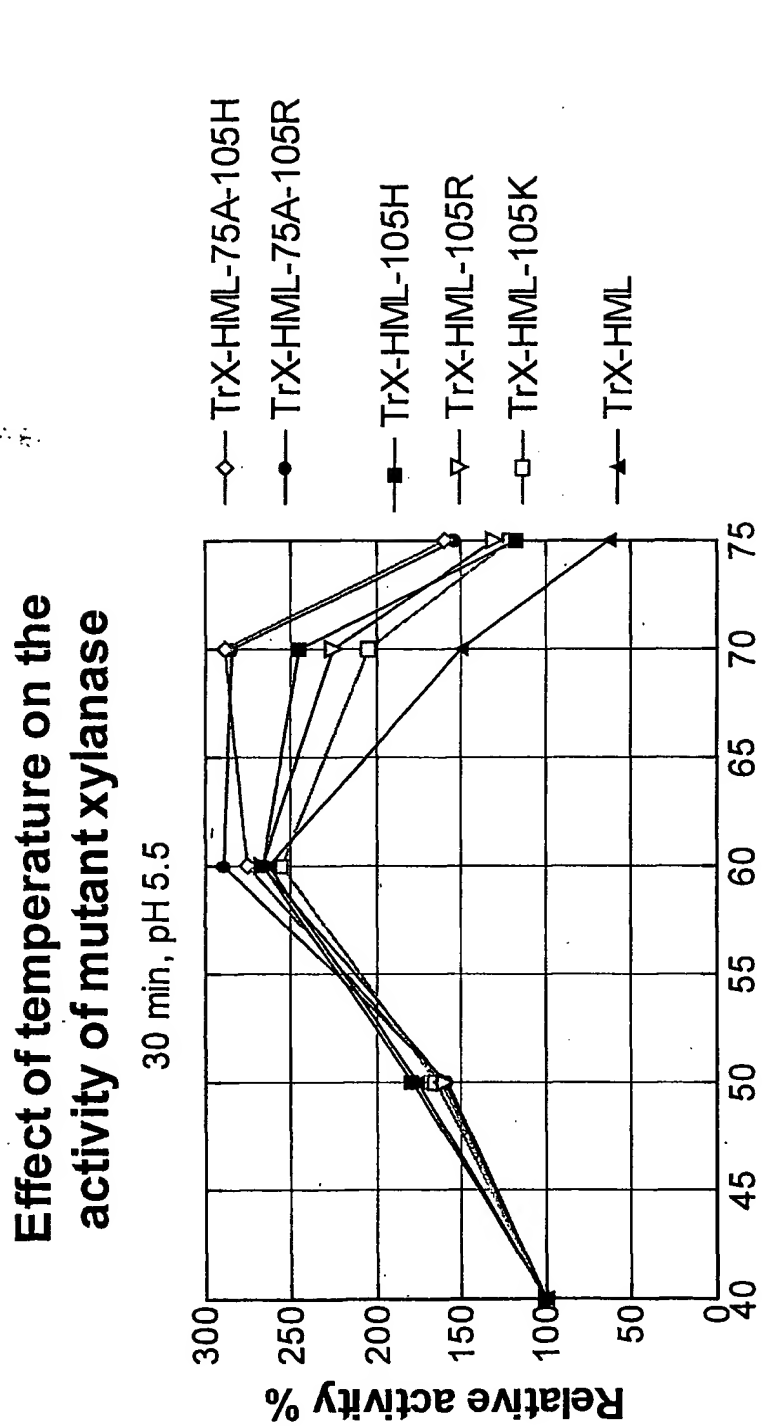
Effect of temperature on the activity of mutant xylanase
30 min, pH 5.5
TrX-HML-75A-105H
TrX-HML-75A-105R
TrX-HML-105H
TrX-HML-105R
TrX-HML-105K
TrX-HML
Relative activity %
300
250
200
150
100
50
0
40
45
50
55
60
65
70
75
Temperature °C

Figure 5

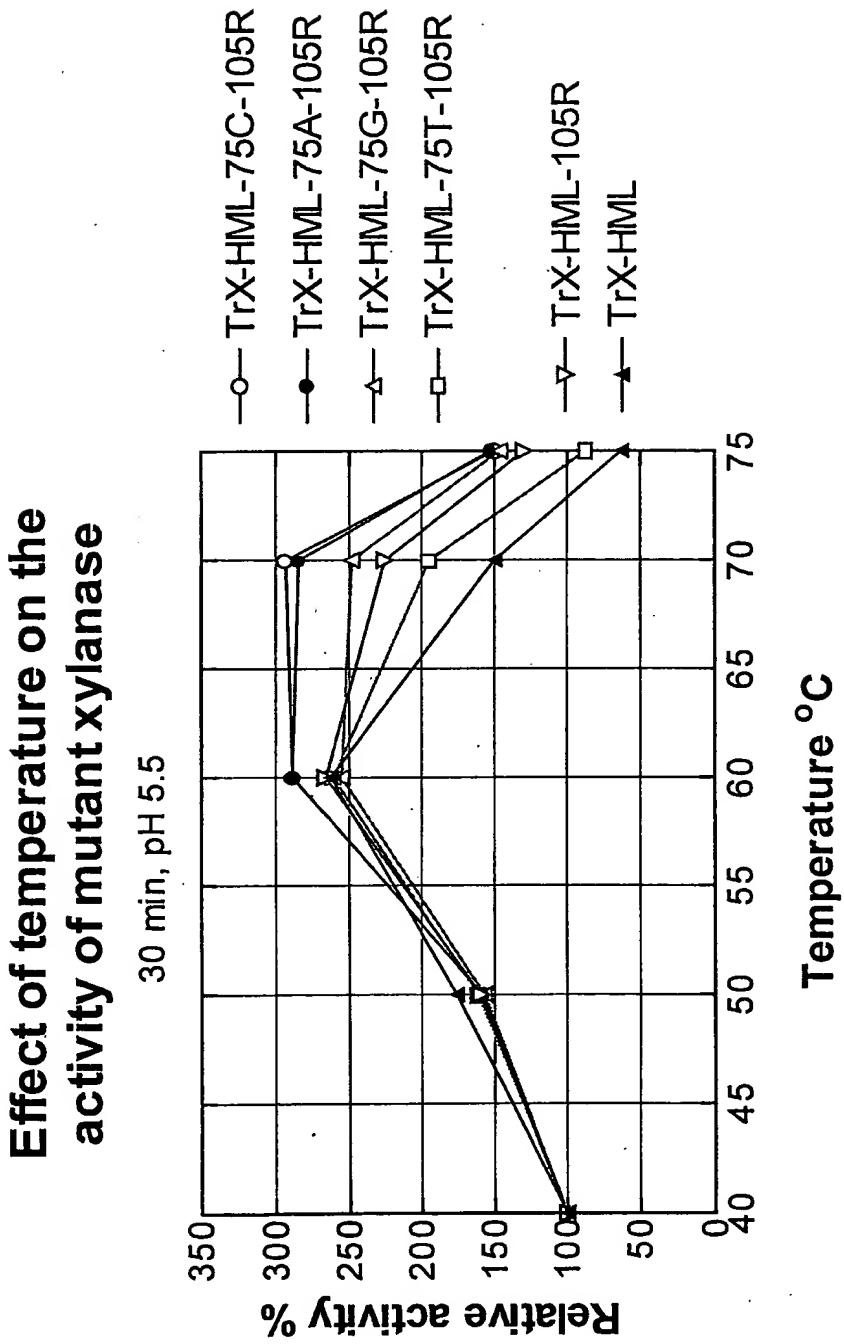
Effect of temperature on the activity of mutant xylanase
30 min, pH 5.5
TrX-HML-75C-105R
TrX-HML-75A-105R
TrX-HML-75G-105R
TrX-HML-75T-105R
TrX-HML-105R
TrX-HML
Relative activity %
350
300
250
200
150
100
50
0
40
45
50
55
60
65
70
75
Temperature °C

Figure 6

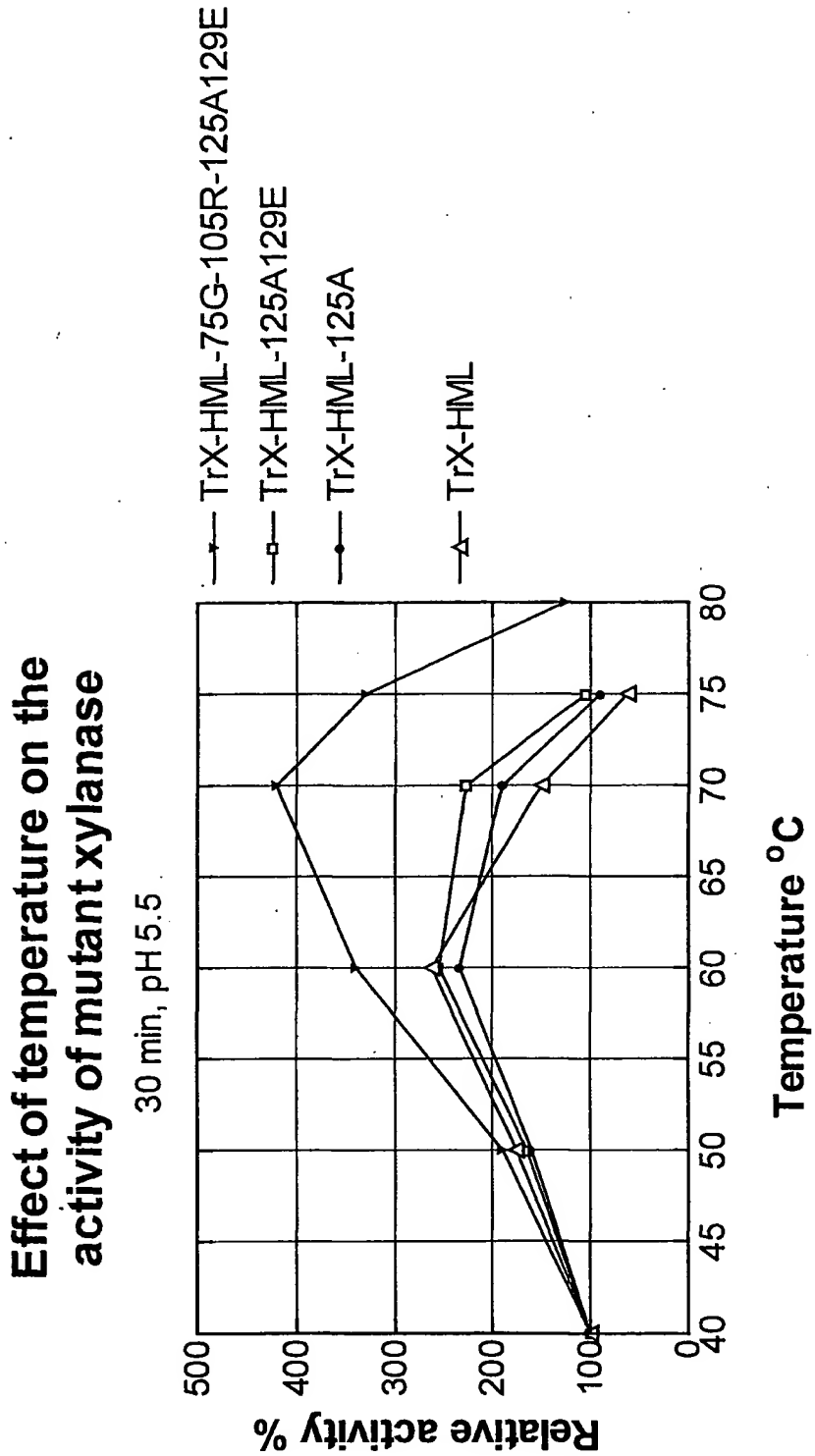
Effect of temperature on the activity of mutant xylanase
30 min, pH 5.5
TrX-HML-75G-105R-125A129E
TrX-HML-125A129E
TrX-HML-125A
TrX-HML
Relative activity %
0
100
200
300
400
500
40
45
50
55
60
65
70
75
80
Temperature °C

Figure 7

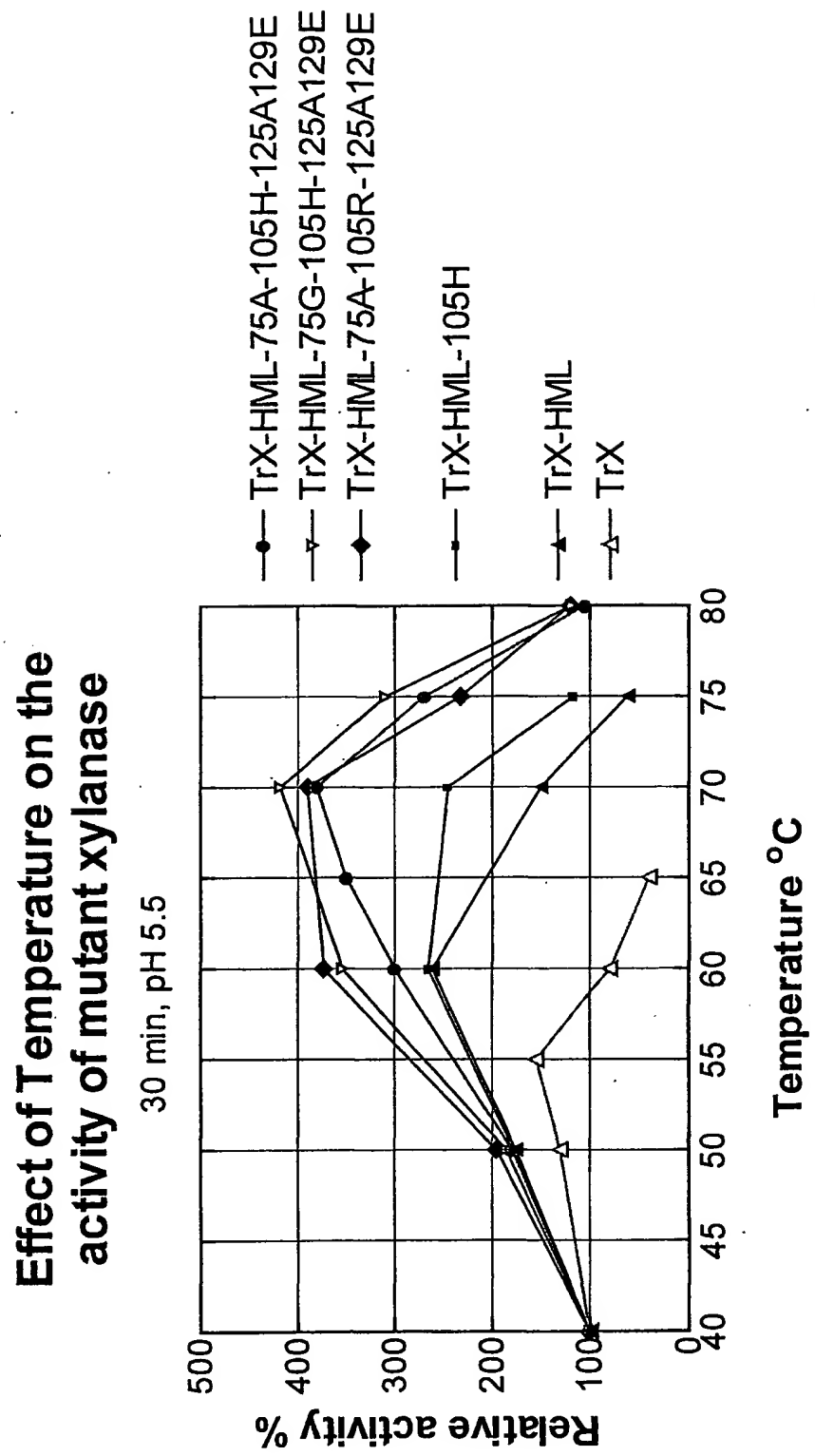
Effect of Temperature on the activity of mutant xylanase
30 min, pH 5.5
TrX-HML-75A-105H-125A129E
TrX-HML-75G-105H-125A129E
TrX-HML-75A-105R-125A129E
TrX-HML-105H
TrX-HML
TrX
Relative activity %
500
400
300
200
100
0
40
45
50
55
60
65
70
75
80
Temperature °C

Figure 8

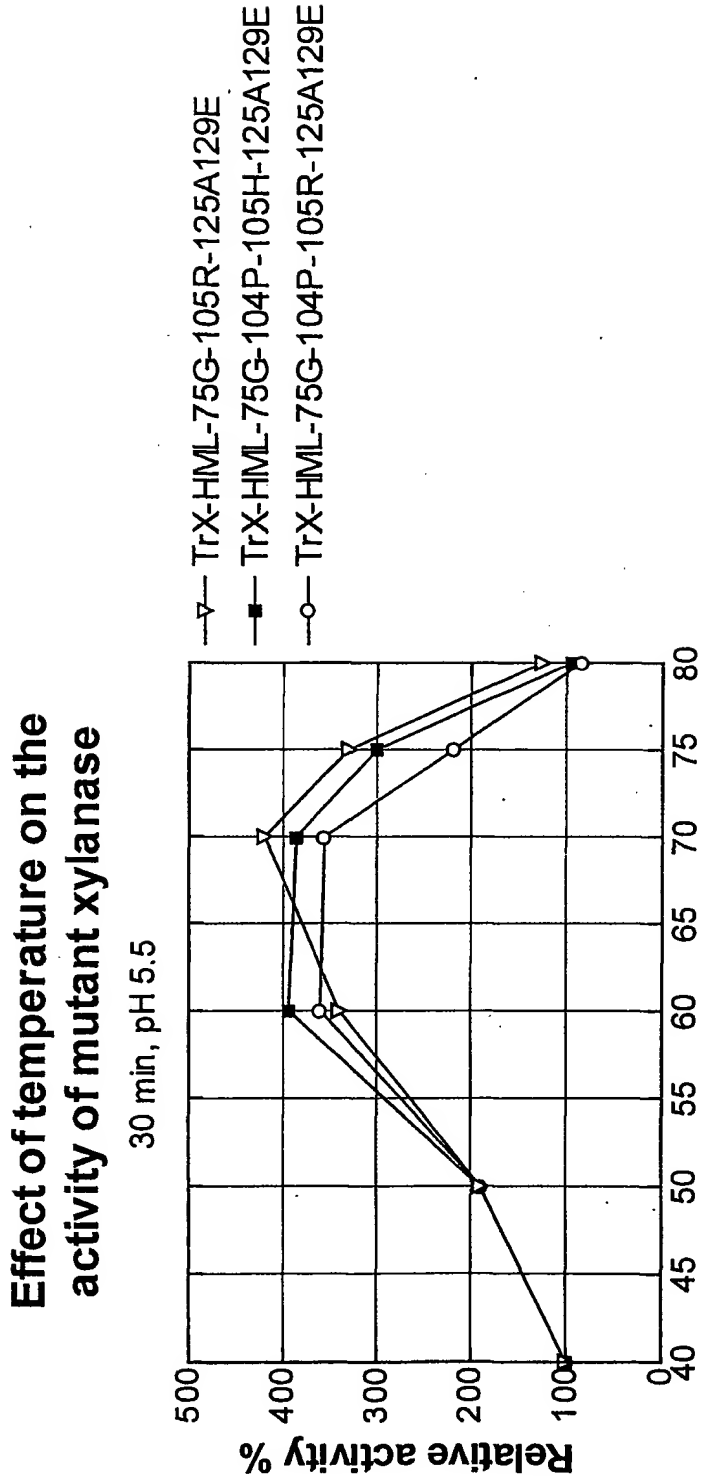
Effect of temperature on the activity of mutant xylanase
30 min, pH 5.5
TrX-HML-75G-105R-125A129E
TrX-HML-75G-104P-105H-125A129E
TrX-HML-75G-104P-105R-125A129E
Relative activity %
500
400
300
200
100
0
40
45
50
55
60
65
70
75
80
Temperature °C

Figure 9

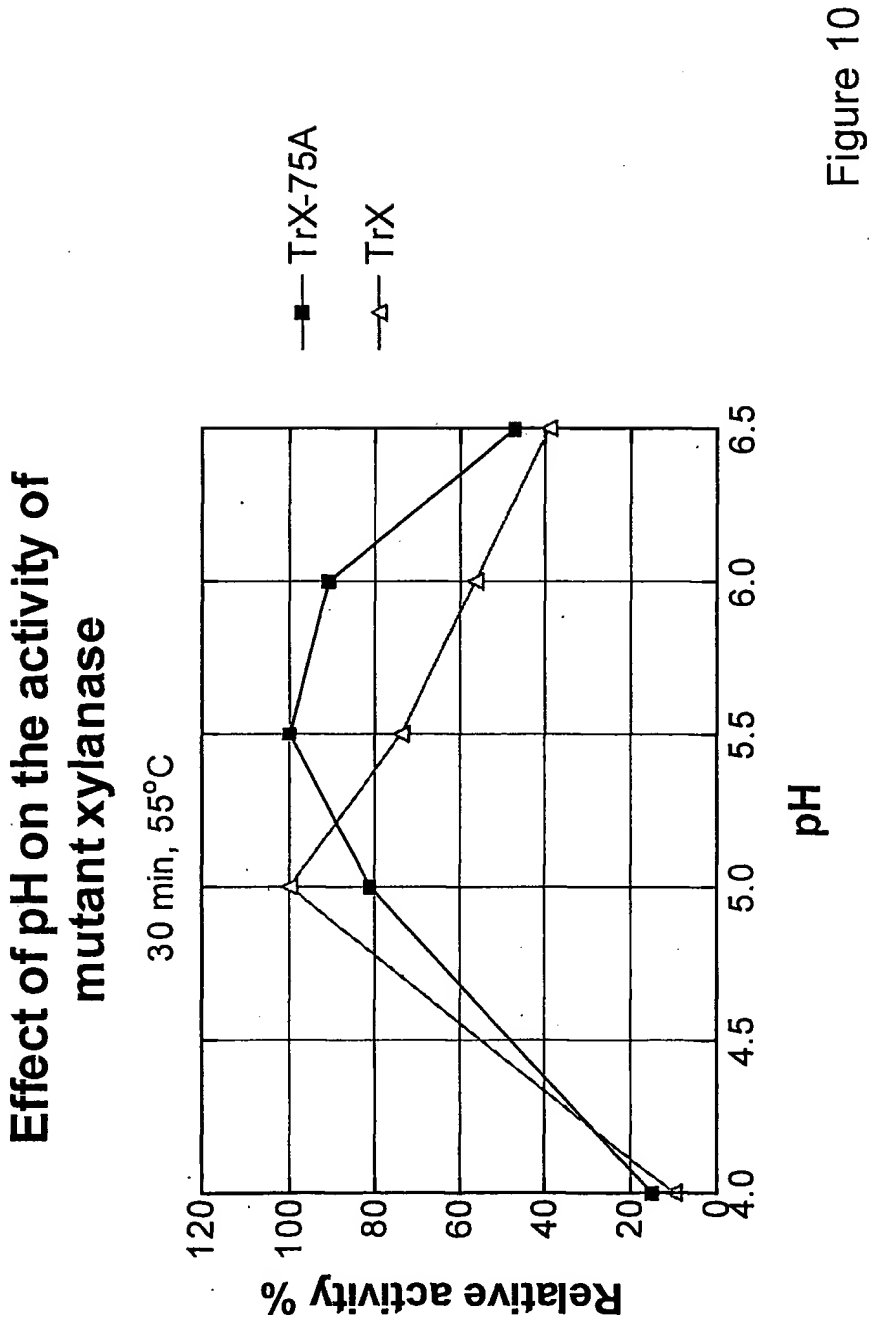
Effect of pH on the activity of mutant xylanase
30 min, 55°C
TrX-75A
TrX
Relative activity %
120
100
80
60
40
20
0
4.0
4.5
5.0
5.5
6.0
6.5
pH

Figure 10

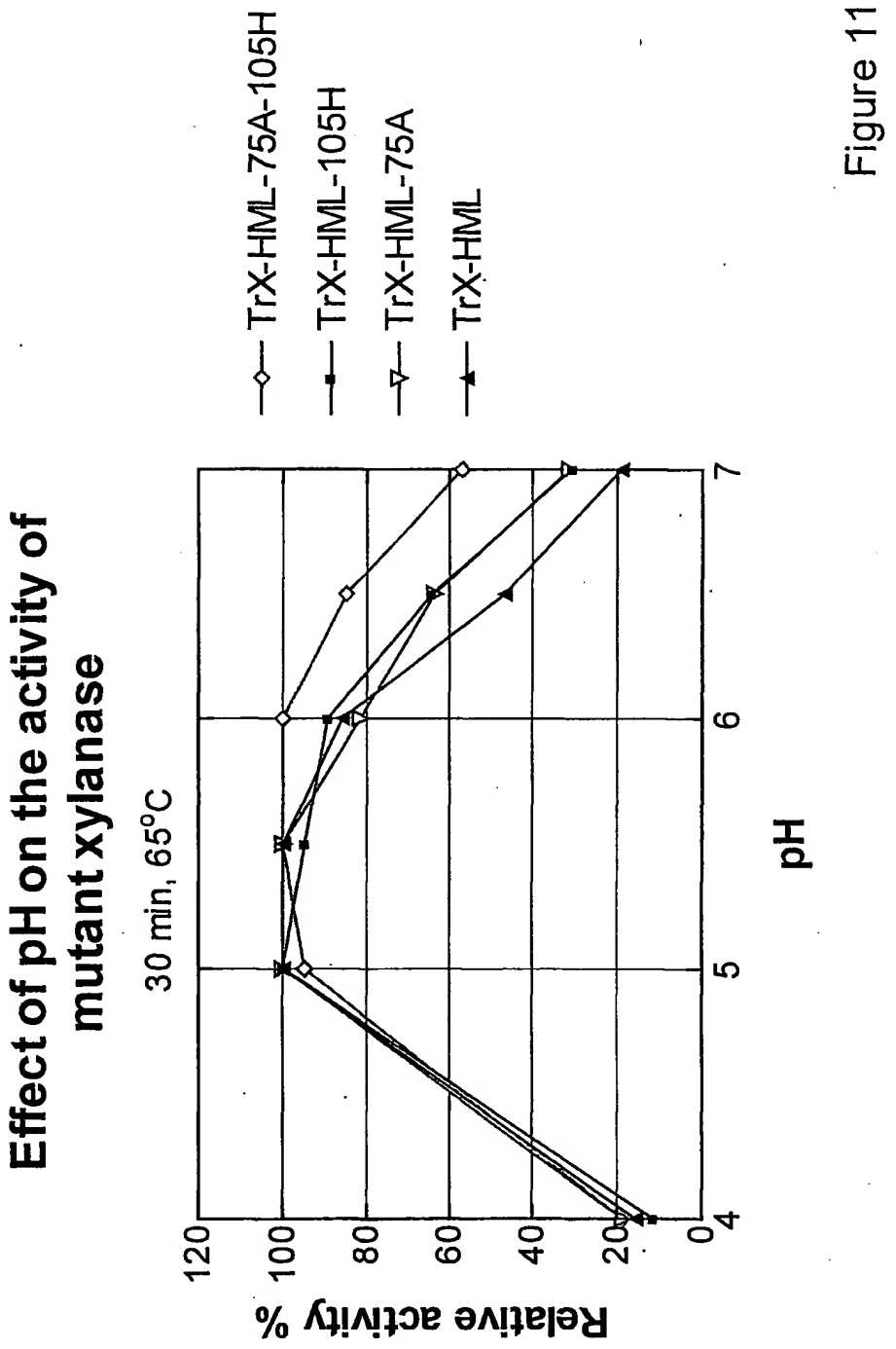
Effect of pH on the activity of mutant xylanase
30 min, 65°C
TrX-HML-75A-105H
TrX-HML-105H
TrX-HML-75A
TrX-HML
Relative activity %
120
100
80
60
40
20
0
4
5
6
7
pH

Figure 11

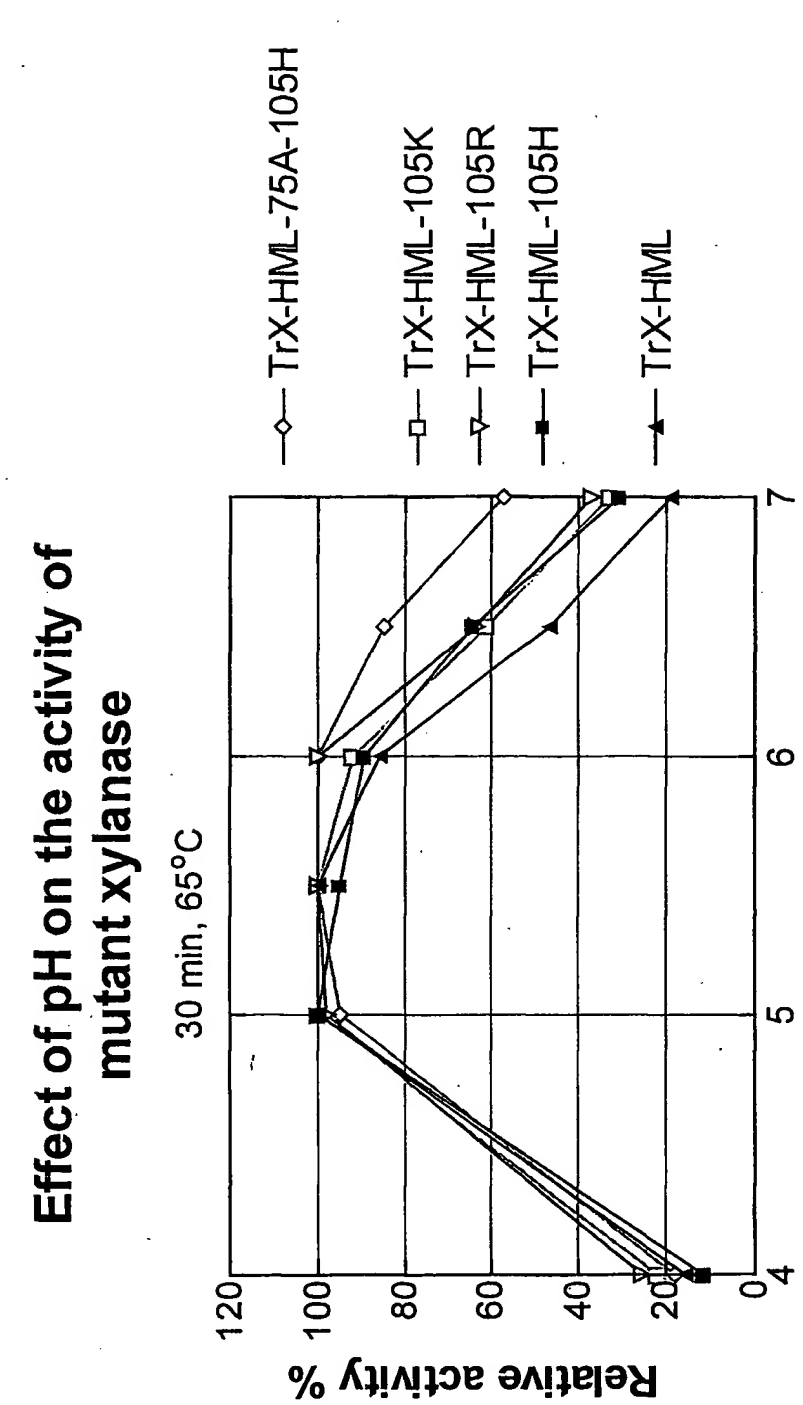
Effect of pH on the activity of mutant xylanase
30 min, 65°C
Relative activity %
120
100
80
60
40
20
0
4
5
6
7
pH
TrX-HML-75A-105H
TrX-HML-105K
TrX-HML-105R
TrX-HML-105H
TrX-HML

Figure 12

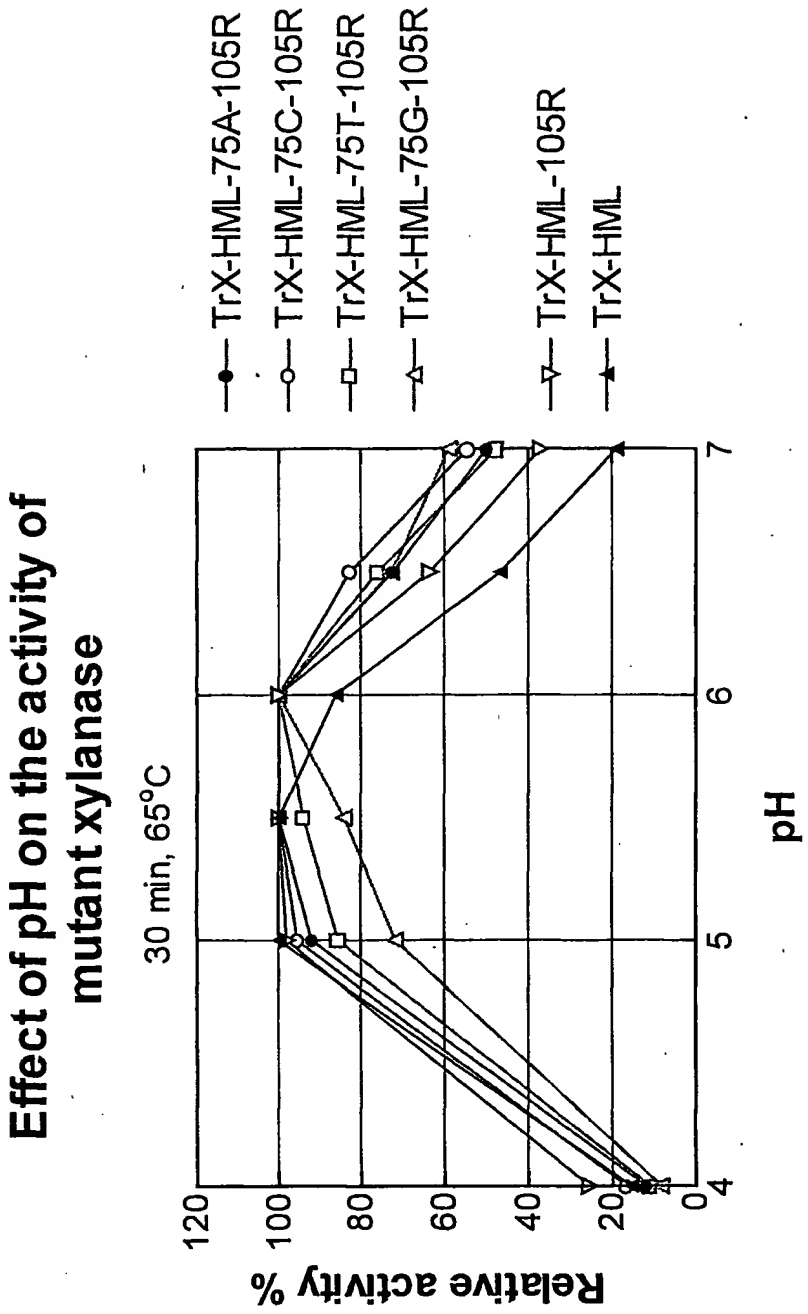
Effect of pH on the activity of mutant xylanase
30 min, 65°C
Relative activity %
120
100
80
60
40
20
0
4
5
6
7
pH
TrX-HML-75A-105R
TrX-HML-75C-105R
TrX-HML-75T-105R
TrX-HML-75G-105R
TrX-HML-105R
TrX-HML

Figure 13

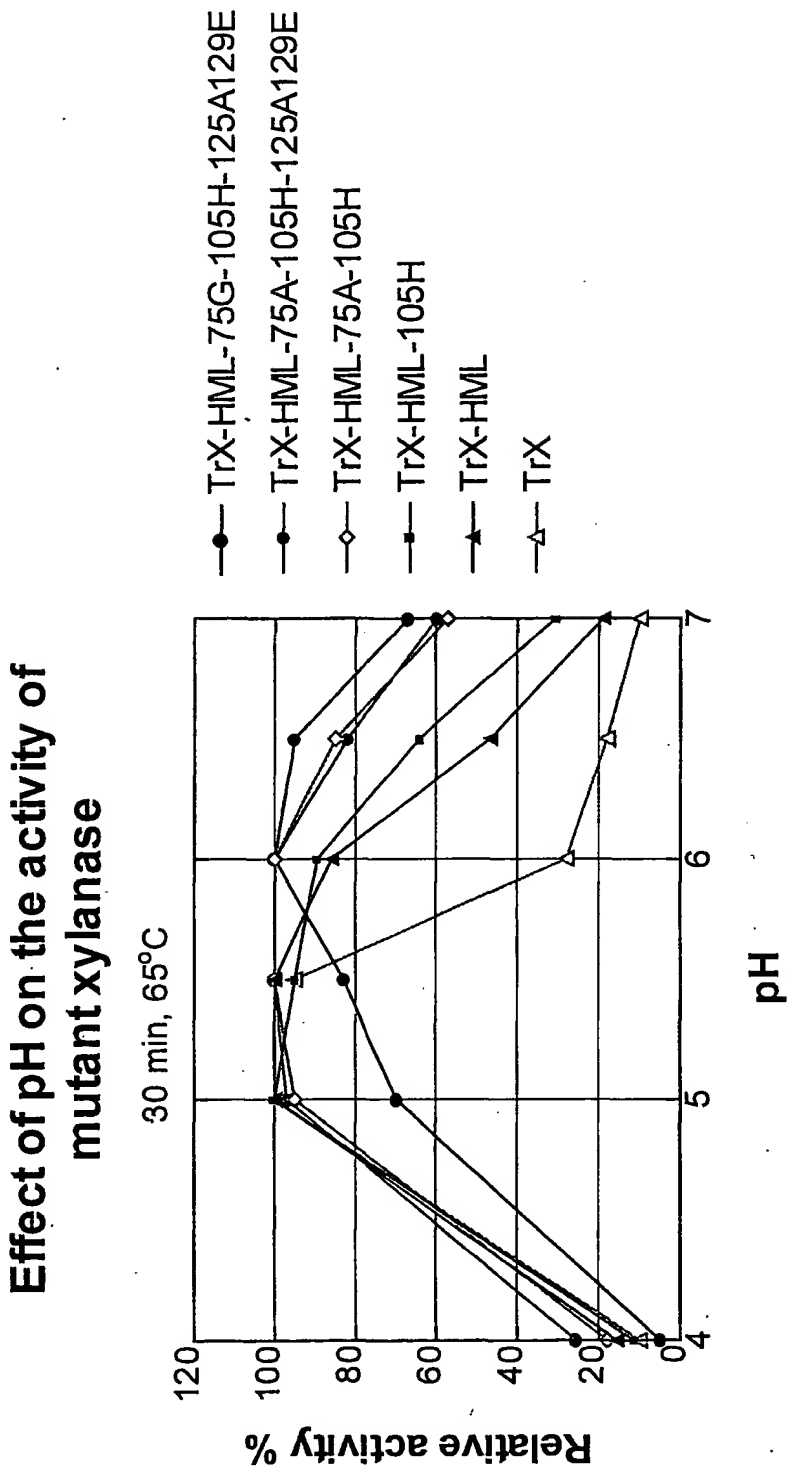
Effect of pH on the activity of mutant xylanase
30 min, 65°C
Relative activity %
120
100
80
60
40
20
0
4
5
6
7
pH
TrX-HML-75G-105H-125A129E
TrX-HML-75A-105H-125A129E
TrX-HML-75A-105H
TrX-HML-105H
TrX-HML
TrX

Figure 14

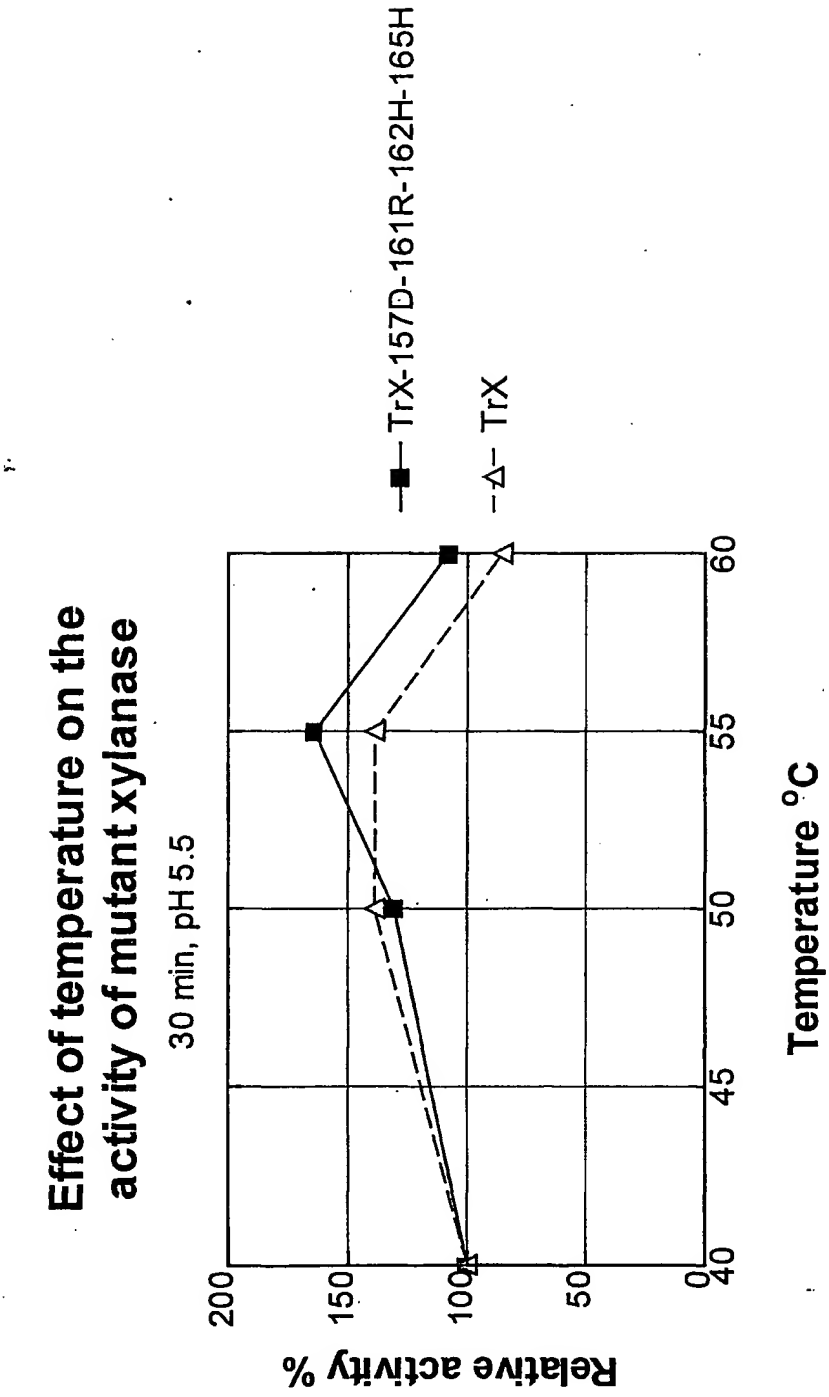
Effect of temperature on the activity of mutant xylanase
30 min, pH 5.5
TrX-157D-161R-162H-165H
TrX
Relative activity %
Temperature °C
200
150
100
50
0
40
45
50
55
60

Figure 15

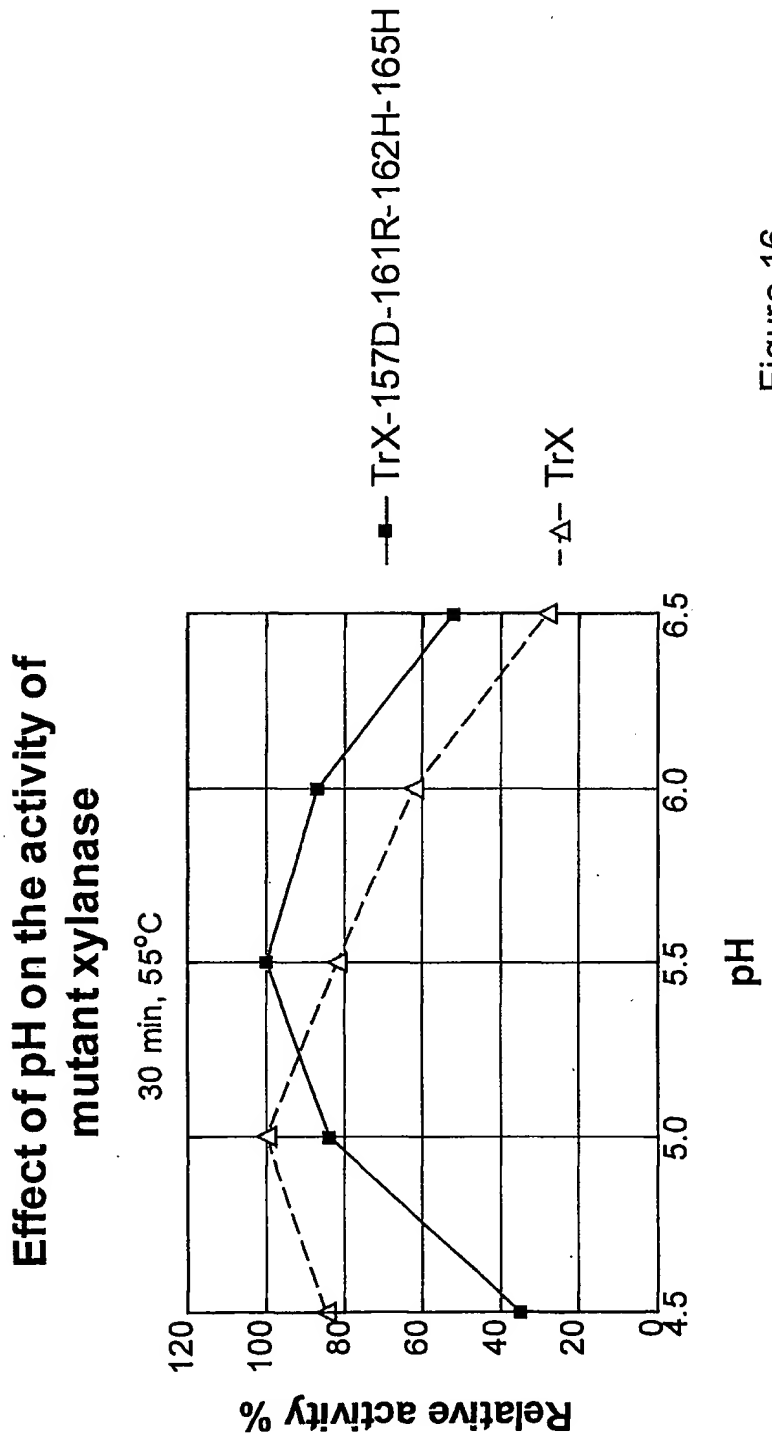
Effect of pH on the activity of mutant xylanase
30 min, 55°C
TrX-157D-161R-162H-165H
TrX
Relative activity %
120
100
80
60
40
20
0
4.5
5.0
5.5
6.0
6.5
pH

Figure 16

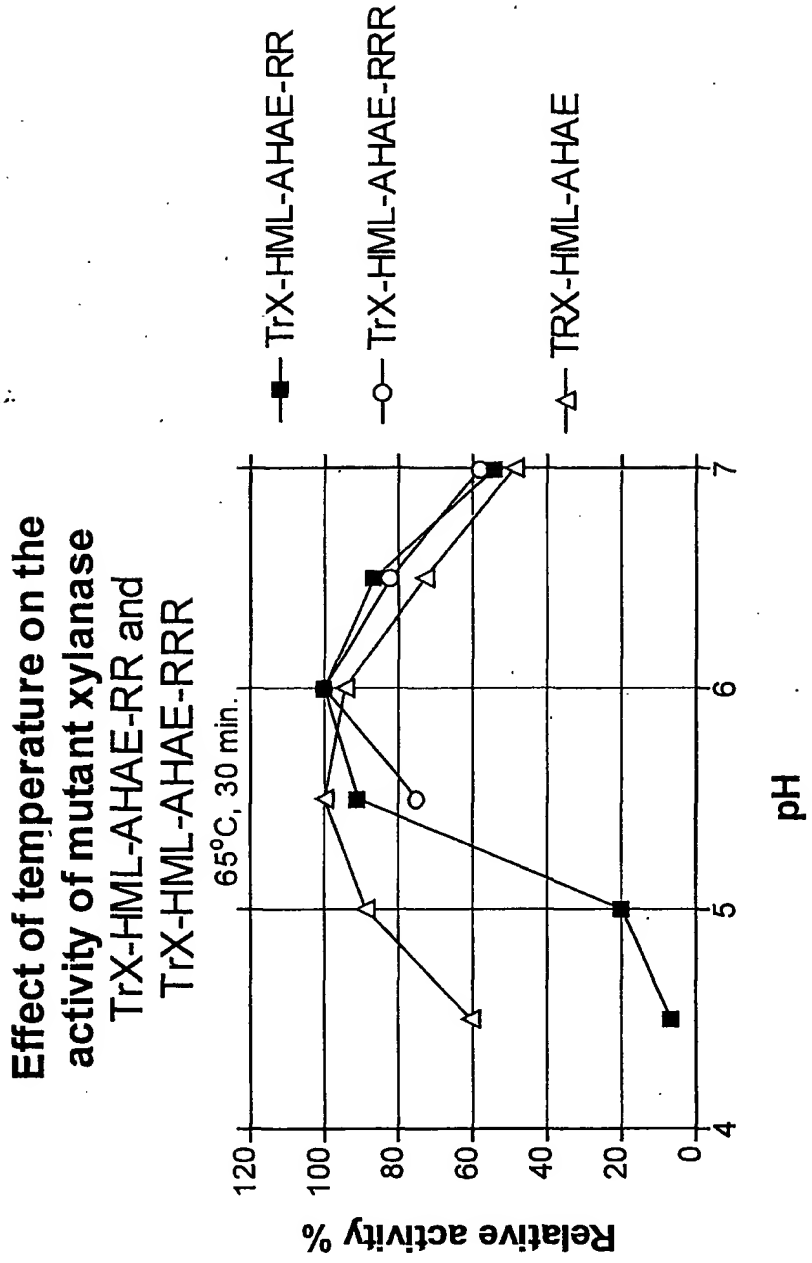
Effect of temperature on the activity of mutant xylanase
TrX-HML-AHAE-RR and
TrX-HML-AHAE-RRR
65°C, 30 min.
TrX-HML-AHAE-RR
TrX-HML-AHAE-RRR
TRX-HML-AHAE
Relative activity %
120
100
80
60
40
20
0
4
5
6
7
pH

Figure 17

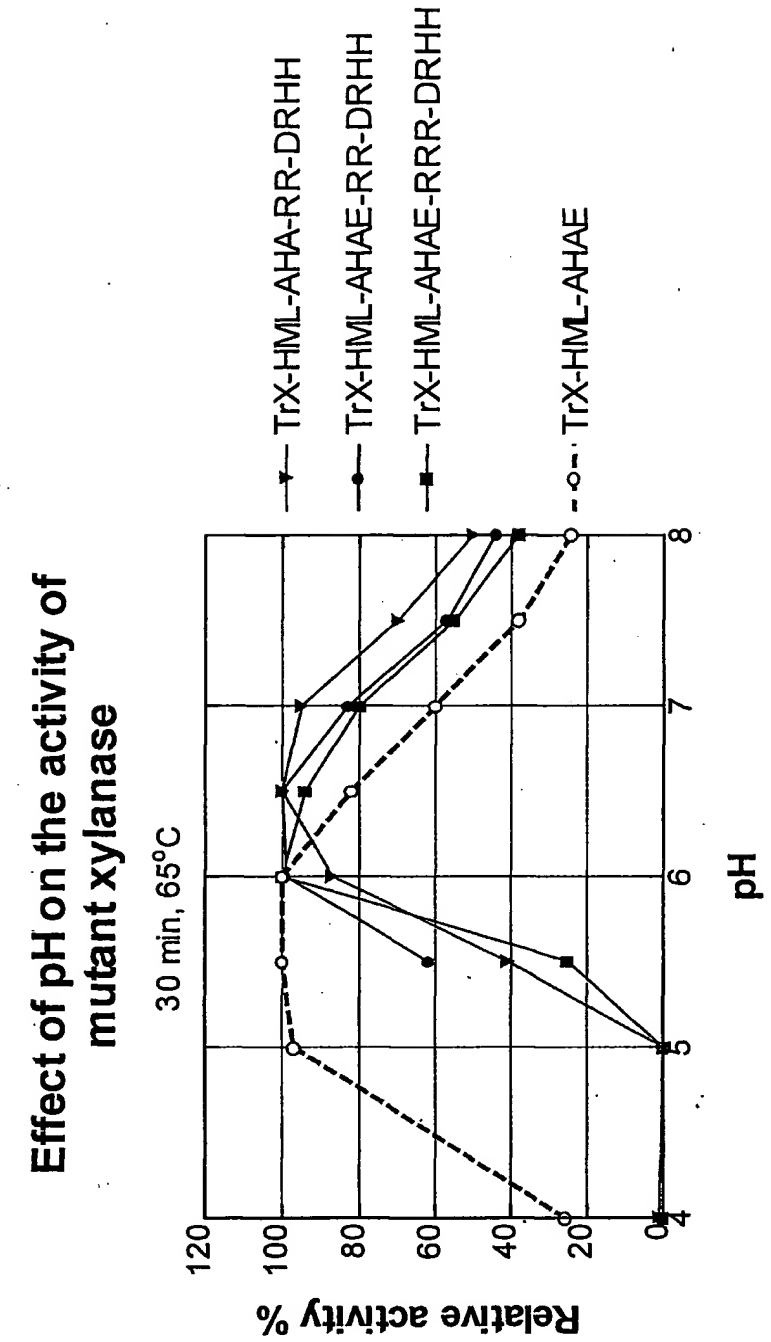
Effect of pH on the activity of mutant xylanase
30 min, 65°C
TrX-HML-AHA-RR-DRHH
TrX-HML-AHAE-RR-DRHH
TrX-HML-AHAE-RRR-DRHH
TrX-HML-AHAE
Relative activity %
120
100
80
60
40
20
0
4
5
6
7
8
pH

Figure 18

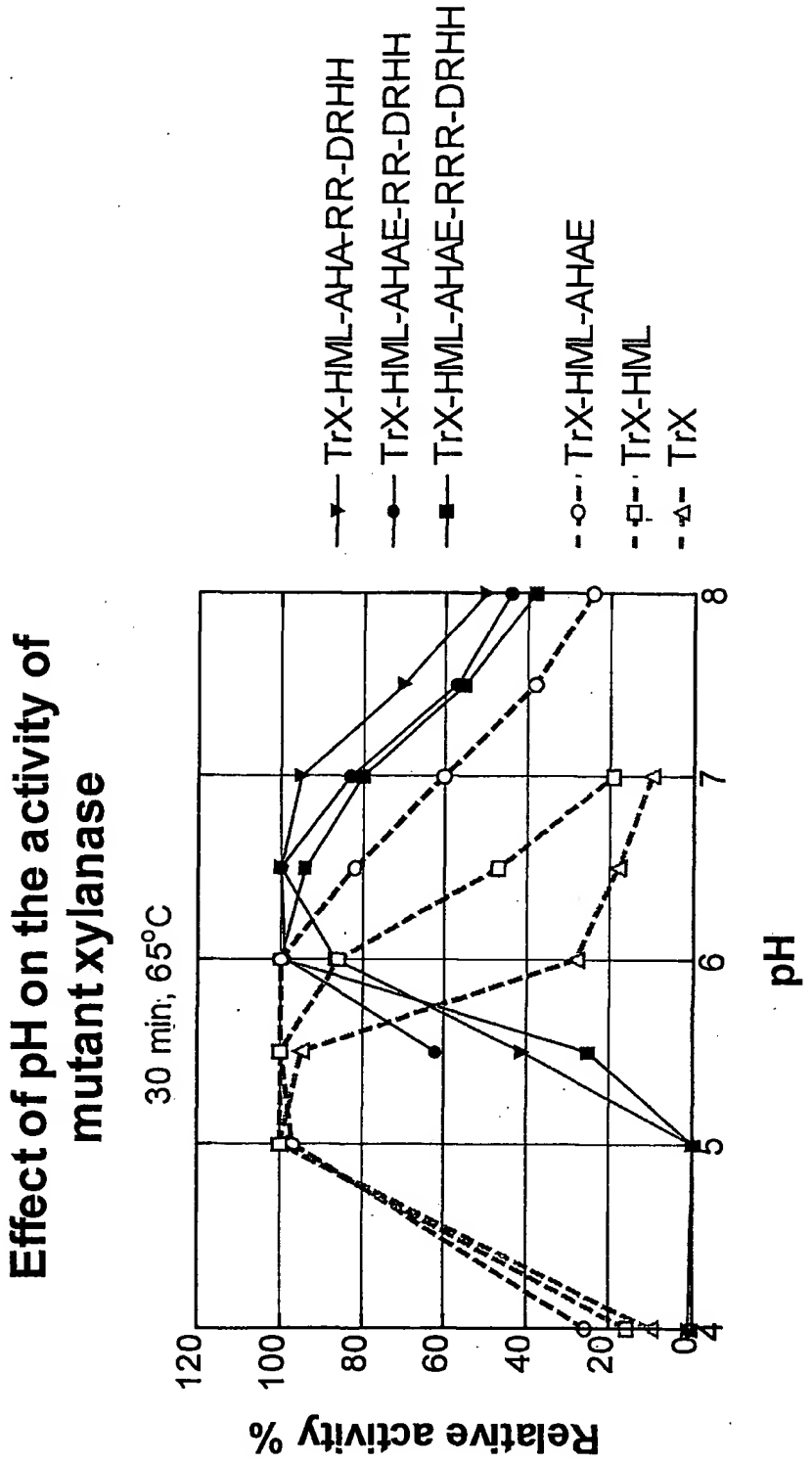
Effect of pH on the activity of mutant xylanase
30 min, 65°C
Relative activity %
pH
120
100
80
60
40
20
0
4
5
6
7
8
TrX-HML-AHA-RR-DRHH
TrX-HML-AHAE-RR-DRHH
TrX-HML-AHAE-RRR-DRHH
TrX-HML-AHAE
TrX-HML
TrX

Figure 19

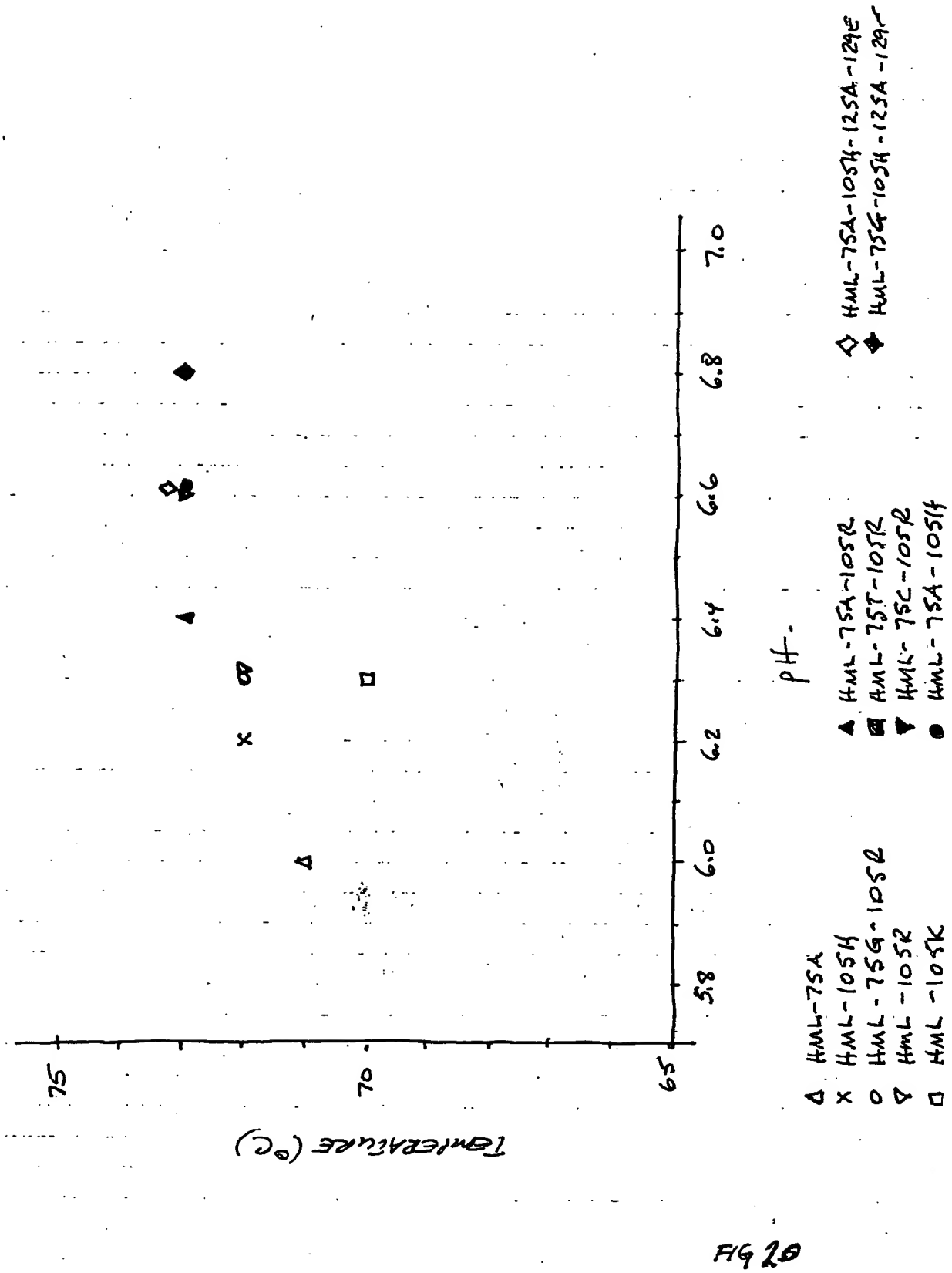
Temperature (°C)
75
70
65
5.8
6.0
6.2
6.4
6.6
6.8
7.0
pH.
Δ HML-75A
× HML-105H
o HML-75G-105R
∇ HML-105R
□ HML-105K
▲ HML-75A-105R
■ HML-75T-105R
▼ HML-75C-105R
● HML-75A-105H
◇ HML-75A-105A-125A-129E
◆ HML-75G-105H-125A-129C

FIG 20